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Research paper

Comparative investigations to evaluate the use of organotypic cultures of transformed and native dermal and epidermal cells for permeation studies

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Abstract

In a comparative study organotypic cultures of both transformed and native dermal and epidermal cells were used for permeation studies. In these organotypic cultures the dermal cells are incorporated into a contracted collagen gel, the epidermal cells are seeded on top of this gel and form a multilayered stratified epidermis when cultivated at the air–liquid interface. For permeation studies two different donor systems containing ibuprofen acid were used, Ibutop-Creme® and Dolgit-Mikrogel®. Studies using excised human stratum corneum showed differences in drug permeability for these two formulations which were also observed when the native organotypic cultures were used. In general, organotypic cultures show a higher permeability for topically applied drugs than excised human stratum corneum, the cultures consisting of transformed cells showed an increase in drug permeability for the Dolgit-Mikrogel® compared with the native cultures which might be due to a higher sensitivity for the enhancer isopropanol in this formulation.

Keywords: Organotypic cultures; Native and transformed dermal and epidermal cells; Permeation studies; Ibuprofen acid

1. Introduction

Since Rheinwald and Green enabled cultivation of human epidermal keratinocytes great progress in cultivation has been made [1]. First cultivation of human keratinocytes was only possible on 3T3 feeder layer, now keratinocytes can be cultivated as a multilayered differentiated epidermis on dermis equivalents resembling the in vivo conditions [2].

It is a common method to use native human keratinocytes for epidermal cultures. This is useful because best approximation of reality will be achieved, but there are also some disadvantages. Native epidermal keratinocytes age rapidly in culture and cell growth stops quickly. Furthermore cultivation over a certain period promotes a selection of cells with the consequence of a change in cell composition [3]. The advantage of using cell lines is the longer lifetime and genetic stability of these cells, but the transformation might be a problem because it could change the properties of the cells such as differentiation functions [3].

The aim of this work was to evaluate and to compare the barrier properties of organotypic cultures of transformed or native cells with respect to the topical delivery of drugs.

In the pharmaceutical sciences normally excised human stratum corneum is used for drug permeation investigations. The disadvantages of excised stratum corneum are the differences in permeability depending on sex, age and nutrition status of the donor and the body site the sample was taken from. Skin equivalents do not have these differences in permeability if culture conditions are standardized.

2. Materials and methods

2.1. Cell cultures

2.1.1. Transformed cell lines

For the cells of the epidermis, the keratinocytes, the

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HaCaT cell line (human adult keratinocytes, low calcium condition, elevated temperature) was used. In contrast to virus transfected cells this cell line is spontaneously transformed (DKFZ, Heidelberg, Germany) [4].

The L-929 cell line was used for the dermal fibroblasts, this is a cell line of murine origin (DSM, Braunschweig, Germany) [5].

Keratinocytes and fibroblasts were cultivated as monolayers using Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% newborn calf serum (Gibco, München, Germany) and 2 mM L-Glutamine (ICN, Eschwege, Germany).

2.1.2. Native cells

Keratinocytes and fibroblasts of native human origin were isolated from skin samples taken from circumcisions according to standard protocols [6]. Fibroblasts were cultivated as a monolayer using DMEM supplemented with 10% newborn calf serum (ICN).

The keratinocytes were cultivated as a monolayer serum-free using a 3:1 mixing of calcium-free DMEM (Biochrom, Berlin, Germany) and Ham's F12 supplemented with adenine (24.3 μg/ml), insulin (5 μg/ml), hydrocortisone (0.4 μg/ml), triiodothyronine (20 pM), transferrin (5 μg/ml) (Sigma, Deisenhofen, Germany), ethanolamine (6.1 μg/ml), phosphoethanolamine (14.1 μg/ml), selenious acid (6.8 ng/ml), strontium chloride (266.6 μg/ml) (Biochrom), epidermal growth factor (EGF, 10 ng/ml), penicillin G sodium (100 U/ml), streptomycin sulfate (100 μg/ml) and amphotericin B (0.25 μg/ml) (Gibco) [6].

By treatment with a solution of 0.1% trypsin and 0.02% versene in phosphate buffered saline (ICN) cell suspensions were obtained. These suspensions were used for the cultivation of the organotypic cultures.

2.1.3. Organotypic cultures

In the organotypic cultures fibroblasts were cultivated in a gel consisting of collagen type I according to the method of Bell et al. [7]. Briefly, a fibroblast cell suspension with a cell concentration of $2–3 \times 10^5$ cells/ml was added to a collagen solution extracted from rat tails. After neutralization with a $NaHCO_3$ solution the collagen formed a gel with incorporated fibroblasts. The gel contraction, caused by the fibroblasts, occurred within 4–7 days.

When gel contraction was finished, the keratinocytes were seeded on top of the dermis equivalent in a concentration of 1.3×10^6 cells/ml. After cultivation submerged for 7 days, the cultures were lifted at the air–liquid interface using Transwell tissue culture inserts (Costar, Fernwald, Germany). For permeation studies, cultures grown for 14 days at the air–liquid interface were used. The medium for the air–liquid cultivation consisted of the medium used for the monolayer cultivation of the human keratinocytes without epidermal growth factor and strontium chloride but supplemented with newborn calf serum (20 μl/ml). To obtain a constant pH during the air–liquid cultivation the tissue culture inserts were placed on sterile cotton pads (Hartmann, Heidenheim, Germany) for a higher medium amount. The cotton pads enabled a doubling of the medium amount from 1.5 to 3 ml.

2.2. Morphological analysis

For histological evaluation the transformed cultures were fixed in 8% formalin, dehydrated in a graded ethanol series and embedded in paraffin. Cross-sections (8 μm) were stained with hematoxylin and eosin for light microscopical examination.

2.3. Immunocytochemical analysis

Immunocytochemistry was performed on 4-μm frozen unfixed cross-sections of the transformed cultures using the indirect peroxidase method. The sections were first incubated with mouse antiserum against the keratins 1, 10 and 11, diluted by 1:100 (MoAb K8.60, ICN) [8], then with the secondary antibody, a peroxidase conjugated antiserum against mouse IgG, diluted by 1:10. After incubation in a diaminobenzidine (0.5 mg/ml)/hydrogen peroxide (0.3 mg/ml) solution (Sigma Fast, Sigma), the sections were examined by light microscopy.

2.4. Permeation studies

For the evaluation of the barrier function of the cultures the upper part of a Franz cell (0.6 cm diameter) was placed on the cultures, which remained in the tissue culture inserts used for cultivation, sealed with a silicone preparation (Baysilone, Bayer AG, Leverkusen, Germany). To allow a greater quantity of solution in the receiver chamber, the tissue culture inserts were lifted by using rubber rings. A schematic representation of this construction is shown in Fig. 1. Permeation studies were performed in triplicate. The permeability coefficients were calculated from the linear ascents of the permeation curves.

The receiver solution contained phosphate buffered saline, pH 7.4, the donors were Ibutop-Creme® and Dolgit-Mikrogel®, both containing 5% ibuprofen acid. Ibutop-Creme® (Deutsche Chefaro Pharma, Waltrop, Germany) is an oil in water cream formulation, Dolgit-Mikrogel® (Dolorgiet, St. Augustin/Bonn, Germany) a cubic liquid crystalline gel.

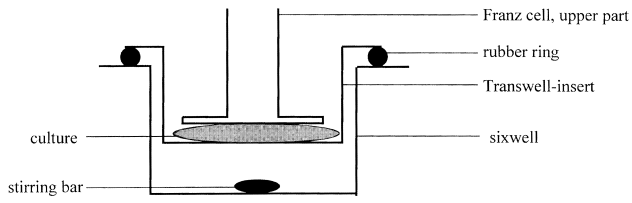


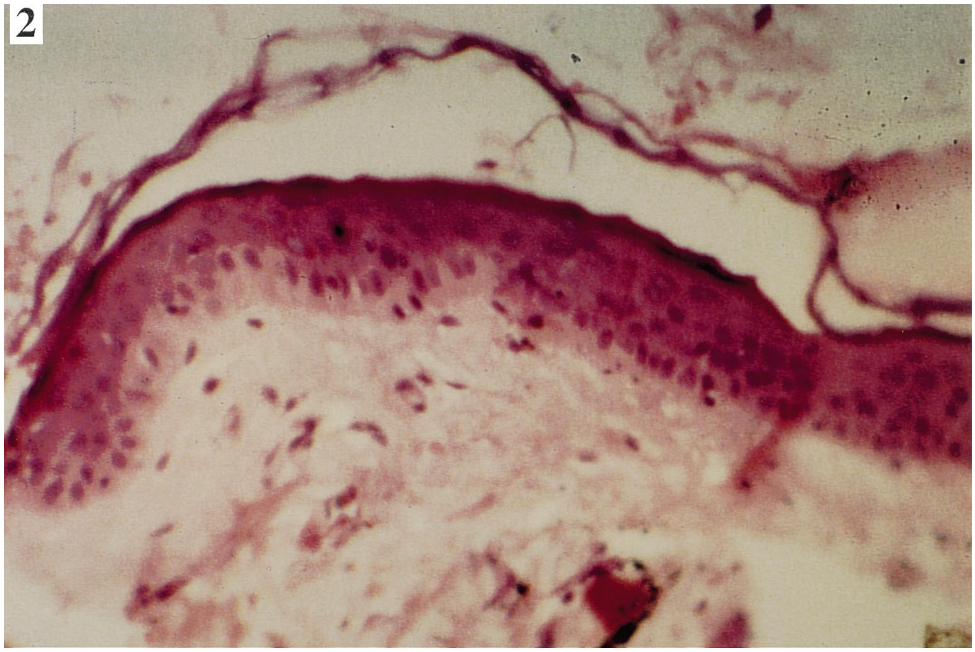
Fig. 1. Schematic representation of the construction used for permeation studies.

Ibuprofen analysis was performed by reversed phase chromatography using a column of Hypersil® ODS 5 μm, 125•4 mm (Grom, Herrenberg, Germany) [9].

The mobile phase consisted of acetonitrile/water/acetic acid (40:60:2) with a flow rate of 1.7 ml/min using a Spectroflow 400 pump (Kratos, Weiterstadt, Germany). Peaks were detected with a Spectroflow 757 absorbance detector (Kratos) at 264 nm, peak identification and integration was carried out by Beckman System Gold Chromatography Software Version 6.01 (Beckman, München, Germany). Calibration was performed within a range of 5–1000 μg/ml with a correlation coefficient of 0.99985.

3. Results and discussion

The HaCaT cells are a spontaneously transformed cell line of human trunk keratinocytes without tumorigenic character [10]. Transplantation of these cells onto mice showed no invasive growth but the development of a



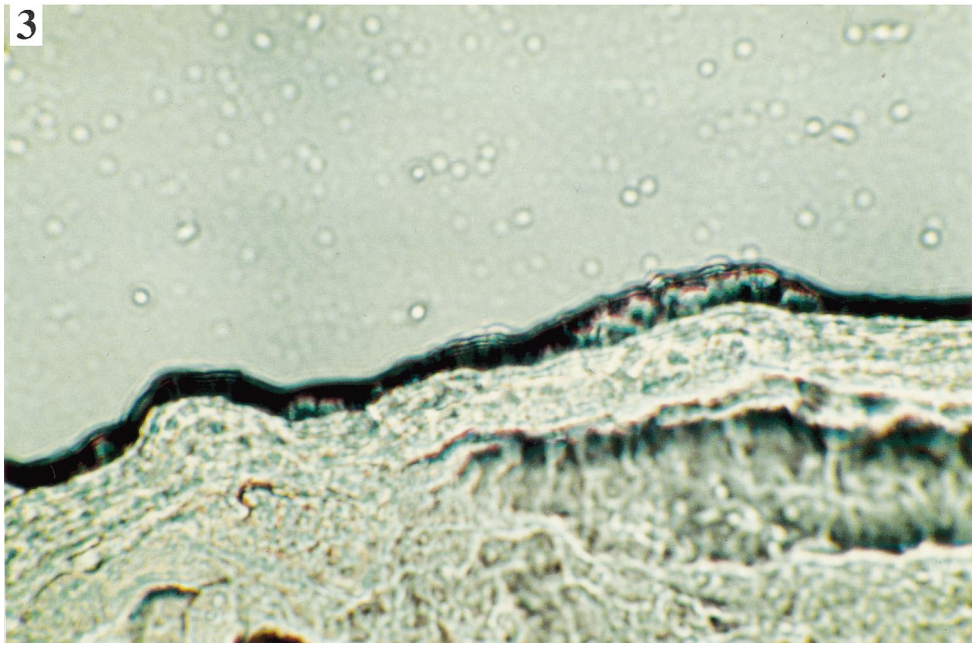


Fig. 2. Cross-section of a transformed organotypic culture (×500).
Fig. 3. Immunohistochemical staining of the cross-section of an organotypic culture of transformed cells (×500).

Table 1

Comparison of the permeability coefficients K_P (cm/s) and standard deviations.

	Native cultures	Transformed cultures	Excised SC [12]
Ibutop-Creme®	$7.17 \pm 1.93 \times 10^{-7}$	$7.62 \pm 1.10 \times 10^{-7}$	3.7×10^{-8}
Dolgit-Mikrogel®	$2.37 \pm 1.07 \times 10^{-6}$	$2.89 \pm 0.21 \times 10^{-6}$	10.97×10^{-8}

fully differentiated, morphologically normal epidermis [10].

For our investigations we used an organotypic epidermal culture of HaCaT cells cultivated on a dermis equivalent consisting of murine L-929 fibroblasts incorporated into a collagen type I gel.

3.1. Morphological analysis

Cross-sections of organotypic cultures of transformed cells cultivated 14 days at the air–liquid interface showed a morphology similar to a native human epidermis (Fig. 2). Distinct cell layers are detectable, and the stratum corneum, the uppermost cell layer, is visible as a multilayered continuous layer.

3.2. Immunocytochemical analysis

To detect the differentiation status of the transformed organotypic cultures immunohistological detection of the differentiation specific keratins 1, 10 and 11 was performed [8]. The antibody used shows low reactivity with keratin 1 and reacts more strongly with the keratins 10 and 11. These keratins are only detectable in a fully differentiated epidermis. Fig. 3 shows the cross-section of an antibody treated organotypic culture. The staining of the upper cell layer is a marker for the presence of these keratins in the epidermis. The appearance of keratins 10 and 11 is restricted to the more superficial outer epidermal layers as it is detectable in organotypic cultures of native epidermal cells [11].

3.3. Permeation studies

The permeation coefficient K_P of ibuprofen acid for the two topical formulations were compared. Differences are detectable between the two formulations for permeation across human excised stratum corneum (SC) [12]. Ibuprofen acid from Dolgit -Mikrogel® permeates human SC three times faster than from Ibutop-Creme® (Table 1). These differences may be attributed to differences in composition of the formulations. Dolgit-Mikrogel® contains isopropanol which is known for its excellent enhancer properties [13].

Permeation of these two formulations across organotypic cultures of native human keratinocytes and fibroblasts generally shows an increase in permeability (Fig. 4). For both formulations the K_P is 20 times higher compared with the permeation data from excised human SC. The permeation of ibuprofen acid from Dolgit-Mikrogel® is still three times faster than from Ibutop-Creme®. Despite the higher permeability of the organotypic native cultures the differences between the two formulations are still detectable in the same ratio as observed in permeation studies across excised SC.

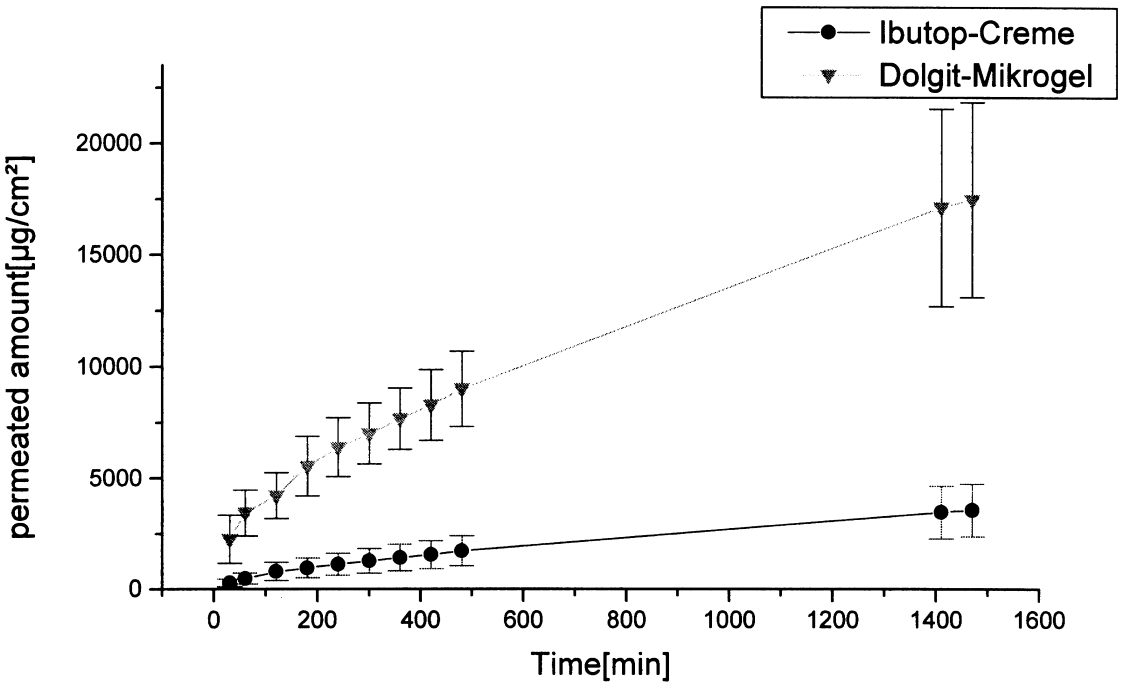


Fig. 4. Permeation of Ibutop-Creme® and Dolgit-Mikrogel® across native organotypic cultures ($n = 3$), graphs represent mean and standard deviation.

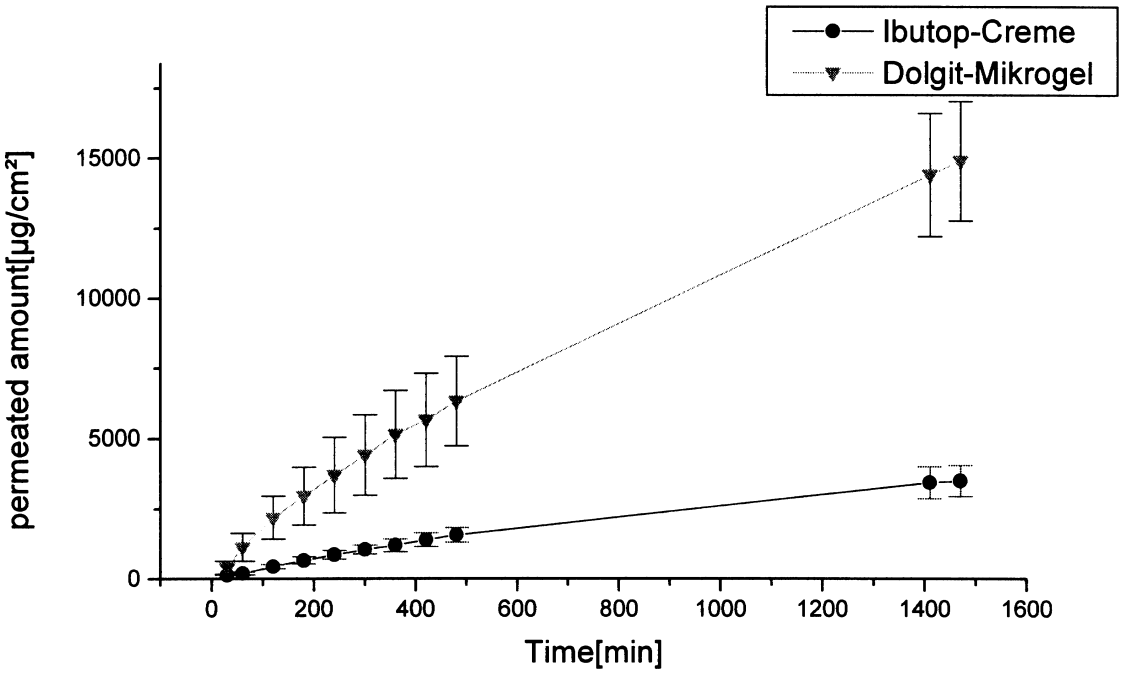


Fig. 5. Permeation of Ibutop-Creme® and Dolgit-Mikrogel® across transformed organotypic cultures (n = 3), graphs represent mean and standard deviation.

The data for permeation across organotypic cultures of transformed cells are the same for Ibutop-Creme® as for the native cultures, but there are differences detectable for the Dolgit-Mikrogel® (Fig. 5). K_P is 1.2 times higher compared with the native cultures and 26 times higher compared with the K_P for excised SC. The Dolgit/Ibutop ratio is here 3.8.

These investigations indicate that organotypic cultures of native or transformed cells have comparable barrier properties. Slight variations are detectable for the permeation of Dolgit-Mikrogel® across the transformed cultures indicating that there are differences between native and transformed cells in forming a barrier function.

The increase in permeability of the transformed cultures using Dolgit-Mikrogel® might also depend on the amount of the enhancer isopropanol in this formulation. A stronger effect of this substance on the barrier function of the HaCaT cells is possible.

4. Conclusion

It is possible to replace human SC in drug permeation studies with organotypic cultures of epidermal and dermal cells. The barrier function of the cultures is less elaborated, but differences in permeation observed for studies using human SC are also detectable. Organotypic cultures of transformed cells are comparable with native cultures, slight differences occur for Dolgit-Mikrogel®. In contrast, permeation of Ibutop-Creme® across both cell culture systems showed identical K_P values. This formulation contains no isopropanol or any other enhancer.

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