International Journal of Pharmaceutics 479 (2015) 422–429

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Effects of topical corticosteroids on cell proliferation, cell cycle progression and apoptosis: *In vitro* comparison on HaCaT

Alexandre Guichard [a,b], Philippe Humbert [a,b], Marion Tissot [b], Patrice Muret [b], Carole Courderot-Masuyer [c], Céline Viennet [b,*]

[a] Research and Studies Center on the Integument (CERT), Department of Dermatology, Clinical Investigation Center (CIC INSERM 1431), Besançon University Hospital, France
[b] Engineering and Cutaneous Biology Laboratory, INSERM UMR 1098, SFR FED 4234, University of Franche-Comte, Besançon, France
[c] BIOEXIGENCE S.A.S., 8 rue Alfred de Vigny, Besançon, France

ARTICLE INFO

Article history:
Received 26 September 2014
Accepted 26 December 2014
Available online 30 December 2014

Keywords:
HaCaT
Keratinocyte
Topical corticosteroid
Proliferation
Apoptosis
Cell cycle

ABSTRACT

Topical-corticosteroids are mainly used for the treatment of inflammatory or hyperproliferative skin diseases. The *in vivo* assay to rank topical-corticosteroids potency, based on the skin blanching, is not adapted to compare their anti-proliferative efficacy. We have compared the antiproliferative effect of six topical-corticosteroids on a model of hyperproliferant keratinocytes (HaCaT). Betamethasone-dipropionate; clobetasol-propionate; betamethasone-valerate; desonide; hydrocortisone-butyrate and hydrocortisone-base, at different concentrations (10^{-8}–10^{-4} M) have been compared. HaCaT proliferation has been evaluated by MTT-assay and the mechanism of the death was evaluated by annexin V/propidium iodide staining and cell cycle phases analysis. Topical corticosteroids reduced cell growth in a dose-dependent manner. At 10^{-4} M, betamethasone dipropionate was the most antiproliferative compound while hydrocortisone-butyrate was the less. Hydrocortisone-base which is usually considered as the less potent topical-corticosteroids showed a clear cytotoxic effect. Betamethasone-dipropionate and betamethasone-valerate induced more apoptosis than necrosis whereas the reverse has been observed for other topical-corticosteroids. All topical-corticosteroids, except clobetasol-propionate, arrested cell cycle mainly in G2-phase. Clobetasol-propionate arrested cell cycle in S-phase population. At 10^{-8} M, topical-corticosteroids induced HaCaT proliferation. In terms of antiproliferative effect at 10^{-4} M, we propose to rank topical corticosteroids as follow: betamethasone-dipropionate > desonide ≥ betamethasone-valerate = hydrocortisone-base = clobetasol-propionate > hydrocortisone-butyrate. This classification differs from the current ranking, based on the vasoconstrictive effect, but is more adapted for hyperproliferative disease treatment.

1. Introduction

Topical corticosteroids (CS) are some of the most widely used drugs in dermatology, mainly for the treatment of inflammatory skin diseases such as psoriasis, atopic dermatitis, contact dermatitis or skin manifestations of auto-immune diseases.

The current standard for measuring topical CS potency is the vasoconstrictor assay or skin blanching assay. This test, described in 1962 by McKenzie and Stoughton, uses the skin pallor, induced at the site of application, as an indicator of the drug potency (McKenzieE and Stoughton, 1962). Indeed, it is proposed that the vasoconstriction is correlated with anti-inflammatory effect and it is the basis for the currently accepted four or seven classes' classification of topical CS. Although, the vasoconstrictor assay remained the gold standard to assess topical CS anti-inflammatory efficacy, this test is probably not adapted to compare topical CS anti-proliferative efficacy. The current topical CS ranking is maybe not correlated with their antiproliferative potency.

The anti-inflammatory, immunosuppressive and anti-proliferative effects of corticosteroids (CS) result from the pleiotropic effects of the glucocorticoid receptor on multiple signaling pathways (Barnes, 1998). The same mechanisms of action, responsible for the improvement of dermatologic inflammatory conditions, can cause adverse effects among which

* Corresponding author at: UFR SMP (Sciences Médicales et Pharmaceutiques), L.I.B.C (Laboratoire d'Ingénierie et de Biologie Cutanée), 19 Rue Ambroise Paré, 25000 Besançon, France. Tel.: +33 363082210; fax: +33 363082260.
E-mail addresses: guichard.alexandre@gmail.com (A. Guichard), philippe.humbert@univ-fcomte.fr (P. Humbert), marion.tissot@univ-fcomte.fr (M. Tissot), patrice.muret@univ-fcomte.fr (P. Muret), bioexigence@wanadoo.fr (C. Courderot-Masuyer), celine.viennet@univ-fcomte.fr (C. Viennet).

http://dx.doi.org/10.1016/j.ijpharm.2014.12.066
0378-5173/

antiproliferative effect in fibroblasts resulting in skin atrophy following prolonged treatment (Ponec et al., 1980; Schäfer-Korting et al., 1996; Ramalingam et al., 1997; Lange et al., 2000; Korting et al., 2002). Several lines of evidence suggest that topical CS inhibit cell proliferation and mitosis and have an inhibitory effect on general protein synthesis of dermal fibroblasts (Hein et al., 1994). Antiproliferative effect of CS results often from a cytotoxic effect, a cell-cycle arrest and an induction of apoptosis (Amsterdam et al., 2002). Cellular viability and cell proliferation have been evaluated with MTT technique for decades. This colorimetric procedure assay is based on the capacity for viable cells to metabolize a tetrazolium colourless salt to a blue formazan which is indirectly correlated to cell viability. Although the MTT assay informs on the percentage of viable cell, the mechanism of the death is evaluated with specific staining. With annexin V and propidium iodide staining, viable and dead cells can be recognized easily using flow cytometry analysis. Cells in early apoptosis with intact plasma membrane integrity are stained only by annexin V, whereas cells in secondary necrosis or late apoptosis, the phase consecutive to apoptosis, contain both stains (Vermes et al., 2000; Kravchenko-Balasha et al., 2009). Besides, cell cycle phases analysis contributes to understand the mechanism of the death by determining the effect of CS on DNA synthesis.

In clinical practice, the efficacy of the topical CS on epidermal proliferative skin conditions, such as psoriasis, is a combination of both anti-inflammatory and anti-proliferative effects on abnormal hyperproliferant keratinocytes. Several *in vitro* studies have shown the anti-inflammatory effect of topical CS on keratinocytes and their antiproliferative effect on fibroblasts, however, none focused on the antiproliferative effect on keratinocytes (Lange et al., 2000, 1997; Zulfakar et al., 2012).

In the current work, we have compared the efficacy of six topical CS, representatives of different potency classes, on the keratinocyte proliferation. Cultured HaCaT, immortalized human keratinocyte line, were used as a relevant experimental model of psoriasis or hyperproliferant skin diseases.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal calf serum (FCS), trypsin-EDTA, phosphate-buffered saline without magnesium and calcium (PBS), penicillin (100 U/mL)-streptomycin (0.1 mg/mL) mixture, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), culture flasks and plates were obtained from D. Dutscher (Brumath, France). Ethanol 100% (Et 100%) was obtained from Sigma–Aldrich (St Quentin Fallavier, France). Annexin V-FITC (AV-FITC) and propidium iodide (PI) were obtained from Cliniscences (Montrouge, France).

Human immortalized non-tumorigenic keratinocyte cell line HaCaT (Caucasian, 62 years, male, skin) was supplied by CLS Cell Lines Service (Germany).

Betamethasone 17,21-dipropionate (BDP); clobetasol 17-propionate (CP); betamethasone 17-valerate (BV); hydrocortisone 17-butyrate (HCB) and hydrocortisone base (HC) were purchased from Sigma–Aldrich (St Quentin Fallavier, France). Desonide (DN) was purchased from Alsachim (Strasbourg, France).

2.2. Preparation of solutions

CS were dissolved in 0.2% of DMSO and 0.5% of Et 100%, added to the DMEM supplemented to obtain stock solutions (10^{-4} M) and then sonicated and filtered (0.2 μm). Different dilutions from 10^{-4} M to 10^{-8} M were extemporaneously obtained from each stock solution. Equivalent amounts of DMSO and Et 100% were used as controls. During experiments, medium without hydrocortisone and bovine pituitary extract was used.

2.3. Cell culture

HaCaT were cultured in 75 cm^2 culture flasks in DMEM medium supplemented with 10% (v/v) FCS and 1% (v/v) of penicillin–streptomycin in a humidified 5% CO_2 atmosphere at 37 °C. The medium was removed every 48 h, until the monolayer cultures reached 70–80% confluency. Cells were then washed with PBS, followed by incubation with trypsin-EDTA at 37 °C for 10 min. The trypsin was deactivated by addition of DMEM supplemented with FCS and the cell suspension centrifuged at 1000 rpm for 5 min. The resulting supernatant was removed and fresh medium added. The suspension was then ready to be used for experimental work.

2.4. MTT cell proliferation assay

HaCaT were seeded into 96-well plates with a density of 5×10^3 cells/well, and were grown with DMEM supplemented for 72 h. Cells were then washed with PBS and 100 μL of each CS or control solutions were added and plates were incubated for 72 h. Medium were then removed and the adhering cells were washed with PBS. MTT solution (0.5 mg/mL in DMEM) was added to the culture wells. After incubation for 4 h at 37 °C, the MTT reaction medium was removed and formazan-blue was solubilized with 100 μL of DMSO. This assay is based on the reduction of the yellow tetrazolium salt MTT by the mitochondrial succinate dehydrogenase to form an insoluble formazan-blue product. Only viable cells with active mitochondria reduce significant amounts of MTT and formazan-blue formation was quantified with a Multiskan FC™ spectrophotometer (Fisher Scientific, Illkirch, France) at 570 nm (Mosmann, 1983).

The number of viable cells was calculated as a percentage of absorbance of the test wells over the corresponding vehicle control (DMSO/Et 100%).

Eight wells were filled for each concentration of CS and vehicle controls. Each experiment was performed five times.

2.5. Apoptosis assay and cell-cycle analysis

HaCaT were seeded in 6-well plates with a seeding density of 5×10^4 cells/well and were grown with DMEM supplemented for 72 h. Medium were then removed and 3 mL of 10^{-4} M or 10^{-8} M solutions of each CS and corresponding vehicle controls were added. Solutions 10^{-8} M and 10^{-4} M were used because they corresponded respectively to the concentration mainly used in other studies (Lange et al., 2000, 1997; Ponec and Kempenaar, 1983; Ponec et al., 1981) and the more concentrated solution we could obtain in our experimental conditions. Plates were then incubated for 72 h to reach 80% confluency. Medium were then removed and the adhering cells were washed twice with PBS and were harvested by trypsinization at 37 °C for 10 min. All supernatant, medium and cells were pooled for the centrifugation and were then separated in two parts for different analysis. One part was fixed and permeabilized with 70% ice-cold ethanol to firstly quantify cell death by flow cytometry as fractions with sub-G1 DNA content and secondly determine cell cycle distribution. Cells were labeled with PI, according to the manufacturer's instructions, followed by flow cytometry (FC500, Beckman Coulter, Villepinte, France).

The other part of cells was used for the quantification of apoptosis and necrosis. Cells were labeled with AV-FITC/PI, according to the manufacturer's instructions, followed by flow cytometry.