Prednicarbate Versus Conventional Topical Glucocorticoids: Pharmacodynamic Characterization *In Vitro*

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Purpose. Pharmacodynamic characterization of topical glucocorticoids as prednicarbate (PC), its metabolites prednisolone 17-ethylcarbonate (PEC) and prednisolone (PD), betamethasone 17-valerate (BMV), betamethasone (BM) and desoximetasone (DM) by evaluating their effects on epidermal and dermal cells. Synopsis of pharmacokinetic and pharmacodynamic studies, possibly explaining the improved benefit–risk ratio of prednicarbate.

Methods. Isolated foreskin keratinocytes were used to investigate the influence on epidermal inflammatory processes, dermal fibroblasts of the same origin to study antiproliferative activities of glucocorticoids. Interleukins were measured by ELISA-assay, the influence on II-1α-production also on mRNA-level by RNAse protection assay. Proliferation was assessed by ^{3}H thymidine incorporation and biodegradation by HPLC/UV-absorption. Cell viability was controlled by MTT assay.

Results. In keratinocytes, inflammation was induced by TNFα, resulting in an increased II-1α synthesis. This cytokine was particularly suppressed by PC and BMV, whereas PEC, PD, DM and BM were less potent ($p \leq 0.05$). Since, however, the double ester PC is rapidly degraded in keratinocytes, a RNAse-protection assay of II-1α mRNA was performed allowing short incubation times and thus minimizing biodegradation effects. In agreement with the previous experiment, the antiinflammatory potency of native PC was confirmed.

In fibroblasts, II-1α and II-6 synthesis indicate proliferation and inflammation respectively. Whereas PC inhibited II-1α and II-6 production in fibroblasts to a minor extent only, it was strongly reduced by the conventional glucocorticoids and PEC ($p \leq 0.05$). The minor unwanted effect of PC on fibroblasts was also reflected by its low influence on cell proliferation as assayed by ^{3}H thymidine incorporation. More pronounced antiproliferative features were observed with BM, PEC and especially BMV.

Conclusions. Correlating antiphlogistic effects in keratinocytes (suppression of II-1α) with antiproliferative effects in fibroblasts (suppression of II-1α and II-6), the improved benefit–risk ratio of PC compared to conventional glucocorticoids does not result only from distinct drug metabolism in the skin but also from a specific influence on the cytokine network.

KEY WORDS: topical glucocorticoids; keratinocytes; fibroblasts; interleukins; benefit-risk ratio.

INTRODUCTION

Investigations in healthy volunteers clearly indicate a separation of the wanted antiinflammatory effects on the skin and the reduction of the dermal thickness with the non-halogenated prednisolone double ester prednicarbate (PC) (1,2). Nonocclusive PC cream did not induce dermal atrophy (3,4) despite its equipotency to betamethasone 17-valerate cream in healthy volunteers (1) and patients with atopic dermatitis (5,6).

The target of our research was to elucidate this clinical result at the molecular level. Furthermore, we wanted to establish in vitro test systems to identify topical glucocorticoids suppressing efficiently epidermal inflammation in keratinocytes without influencing fibroblast proliferation and thus causing dermal atrophy. In the future, these test systems will help to reduce animal experiments during drug development.

Important pharmacodynamic effects of PC and its metabolites were derived from the influence on cytokine synthesis and cell proliferation in isolated human foreskin keratinocytes and fibroblasts. Cytokines like II-1α and II-6 are constitutively synthesized by keratinocytes and fibroblasts. Especially the production of II-1α and II-6 increases transiently following proinflammatory stimuli such as TNFα (7,8) by phosphorylation and thus inactivation of the inhibitory transcription factor IκBα. In turn, the transcription factor NFκB is released and induces the expression of a wide range of cytokine genes. Glucocorticoids inhibit transcription of cytokine genes by inducing IκBα production (9,10) or by directly interacting with NFκB (11).

In keratinocytes, II-1α is formed and partly released following inflammatory stimuli (12,13). In fibroblasts, however, II-1α is responsible for proliferation, collagenase induction and II-6 synthesis, the latter appears to be associated with fibroblast inflammation (8,14). Inhibition of II-1α synthesis in keratinocytes thus indicates antiphlogistic potency, whereas in fibroblasts antiproliferative effects have to be expected.

Finally, correlation of beneficial effects (II-1α suppression in keratinocytes) with the atrophogenic risk (II-1α-, II-6-suppression, inhibition of ^{3}H thymidine incorporation in DNA of fibroblasts) may help to evaluate the therapeutic value of a glucocorticoid applied for inflammatory skin disease.

MATERIALS AND METHODS

Solutions and Preparations

PC, PEC, and PD were obtained from Hoechst AG (Frankfurt a.M., FRG). DM, BMV, BM, trypsin, sodium-EDTA,

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ABBREVIATIONS: BM, betamethasone; BMV, betamethasone 17-valerate; BPE, bovine pituitary extract; DM, desoximetasone; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethylsulfoxide; FCS, fetal calf serum; hEGF, human epidermal growth factor; hGAPDH, human glyceraldehyde-3-phosphate dehydrogenase; II-1α, interleukin 1 alpha; II-6, interleukin 6; KBM, keratinocyte basal medium; KGM, keratinocyte growth medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; PBS, phosphate buffered saline; PC, prednicarbate; prednisolone 17-ethylcarbonate, 21-propionate; PD, prednisolone; PEC, prednisolone 17-ethylcarbonate; RT-PCR, reverse-transcription-polymerase chain reaction; SDS, sodium dodecylsulfate; TBE, tris-borate-EDTA-buffer; TEMED, N,N,N′,N′-tetramethyl-ethylenediamine; TNF α, tumor necrosis factor alpha.

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trichloracetic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT), phosphate buffered saline (PBS), glutamine, and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma (Deisenhofen, FRG). TRIzol® and fetal calf serum (FCS) was acquired from Gibco BRL, Life Technologies (Eggenstein, FRG), and tumor necrosis factor α (TNFα) from Seromed Biochrom (Berlin, FRG). Scintillation fluid was obtained from Wallac (Freiburg, FRG) and ^{3}H thymidine from ICN (Eschwege, FRG).

Keratinocyte basal medium (KBM), bovine pituitary extract (BPE), human epidermal growth factor (hEGF), insulin, amphotericin B, gentamicin sulfate, and hydrocortisone were purchased from Clonetics (San Diego, California). Dimethylsulfoxide (DMSO), acetic acid, and sodium dodecylsulfate (SDS) were acquired from Merck (Darmstadt, FRG). ELISA kits for II-1α and II-6 were purchased from Coulter-Immunotech (Hamburg, FRG) and a ribonuclease protection assay kit from AMBION (Austin, Texas). Non-denaturing gel (15 ml, sufficient for 1 gel) consisted in 1.5 ml TBE, 2.5 ml 30% acrylamide (19:1), 11 ml dH_2O, 120 μl ammonium persulfate in dH_2O, and 16 μl TEMED, ingredients were obtained from Biometra (Göttingen, FRG). Gel was analyzed and quantified by the phosphoimager system from Fuji (Düsseldorf, FRG). Glucocorticoid stock solutions (10^{-3} M) in ethanol (96%) kept at −80°C were stable for at least 3 months. Stock solutions were diluted in medium immediately before the experiment. Growth medium for keratinocytes was serum-free KBM, supplemented with hydrocortisone (0.5 μg/ml), BPE (30 μg/ml), hEGF (0.1 ng/ml), insulin (5 μg/ml), amphotericin B (50 ng/ml), and gentamicin sulfate (50μg/ml). During experiments medium without hydrocortisone and BPE was used. Fibroblasts were grown in DMEM, supplemented with FCS (10%), glutamine (0.2 nM), amphotericin B (50 ng/ml), and gentamicin sulfate (20 μg/ml).

Cell Culture

Human juvenile foreskin biopsies were incubated at 4°C in a 0.25% trypsin/0.1% EDTA solution for 20 h. Trypsinization was stopped by ice-cold PBS, and keratinocytes were scraped off from the skin surface by forceps, filtered through sterile gauze, centrifuged (5 min/1000 rpm) and resuspended in medium. The keratinocytes were seeded into prewarmed medium and incubated at 37°C/5% CO_2. The remaining skin was trypsinized for another 10 min at 37°C. The enzymatic reaction was stopped by fibroblast medium, and the fibroblasts were scraped off from the dermis. As mentioned before, cells were filtered, centrifuged, resuspended, seeded into fibroblast medium and incubated at 37°C/ 5% CO_2.

Stimulation of Interleukin Synthesis

Pooled keratinocytes (at least two donors) of the 2nd or 3rd passage and pooled fibroblasts of the 2nd–7th passage were used. MTT assays (15) accompanied each experiment to correct results for cytotoxic effects if necessary.

For cytokine experiments, 10^5 cells were seeded per well of a six-well plate. Plates were incubated for 24 h at 37°C/5% CO_2. II-1α and II-6 production was induced by 600 U TNFα (in PBS). After a period of 10 min, glucocorticoid solutions were added to a final concentration of 10^{-8} M. Plates were shaken with 350 rpm for 10 min to allow appropriate distribution.

ELISA: Measurement of II-1α Synthesis

After 24 h the supernatant was removed and remaining cells were lysed in 0.1% EDTA-solution (in PBS) by repeated freeze-thawing cycles. The probes remained frozen at −80°C until used in the ELISA. II-1α and II-6 were quantified by selective ELISA-kits following the instructions of the manufacturer. Samples were analyzed and quantified by an ELISA-reader from Merlin (Bornheim, FRG) at 405 nm. Detection limits were 3 pg II-6/ml and 5 pg II-1α/ml. The antibody of II-1α crossreacted with the active II-1α precursor but not with II-1β. TNFα stimulated control was set 100%.

RNAse Protection Assay: Measurement of II-1α mRNA

For RNAse protection assay, 3 h after stimulation by 600 U TNFα and subsequent glucocorticoid treatment (10^{-8} M) supernatants were removed and incubation was stopped by 1 ml TRIzol®. 0.2 ml Chloroform per tube was added, the tubes shaken and centrifuged (12,000×g/15 min 4°C). Following centrifugation, the aqueous phase was transferred to a fresh tube and RNA was precipitated by 0.5 ml isopropyl alcohol. After incubation for 10 min and subsequent centrifugation (12,000×g/10 min 4°C), the RNA pellet was washed with 1 ml ethanol (75%), vortexed, and centrifuged (7,500×g/5 min/4°C). After the procedure, the RNA pellets were briefly dried (10–15 min) and redissolved in RNAse free water.

The RNA was used for RNAse protection assay with probes for the human II-1α and human glyceraldehyde-3-phosphate-dehydrogenase (hGAPDH) genes isolated by reverse-transcription-polymerase chain reaction (RT-PCR) from human keratinocyte RNA. The II-1α probe corresponds to nucleotide 87 to 307 in the sequence published by Nishida (16). The hGAPDH probe was prepared with the primers described by Wong (17) and corresponds to nucleotides 51 to 234 of hGAPDH-mRNA (18). RNAse protection assays were performed with a commercially available Kit according to the manufacturers instructions. Briefly, 45,000 cpm of both labeled RNA-probes were coprecipitated with the cellular RNA, resuspended and hybridized overnight at 45°C. Unbound probe was digested by RNAses A and T1 and the protected portion was electrophoretically analyzed and quantified using a phosphoimager system. The intensity of the II-1α signal was normalized to the one of the hGAPDH. Control was stimulated by 600 U TNF without the application of glucocorticoids and was set 100%.

^{3}H Thymidine Assay

10^4 fibroblasts were seeded per well of a 24-well plate. After incubation for 24 h growth medium containing 10^{-5} M or 10^{-6} M of the respective agent was dispensed into the wells and cells were incubated for 48 h. Cells were allowed to incorporate radioactive ^{3}H thymidine (1 μCi / well) for another 23 h. After a total incubation time of 72 h, cells were washed twice with PBS, and twice with ice-cold trichloracetic acid (3%) to remove excessive radioactivity. Cells were lysed by 200 μl of 0.3 N NaOH and shaken for 1 h/300 rpm. 100

μL of each aliquot were mixed thoroughly with 1.5 ml of scintillation fluid.

MTT Test

Cell viability was measured by MTT test as described elsewhere (15). Quantification was realized by an ELISA-reader from Merlin (Bornheim, FRG) at 590 nm.

Drug Metabolism

PC metabolism was determined as described previously [19].

Statistics

Arithmetic mean values and standard deviation (SD) of at least triplicate analysis were calculated. Significance of differences as compared to glucocorticoid-free control and to PC treatment was analyzed using Shapiro-Wilk-test, F-, and Student's t-test. If necessary, Welch-test and Dixon-test were applied. A p value ≤ 0.05 was considered significant.

RESULTS

Antiphlogistic Effects of Topical Glucocorticoids in Isolated Keratinocytes

To evaluate the antiinflammatory effects of glucocorticoids, we measured the glucocorticoid dependent decline of TNFα stimulated II-1α synthesis which turned out to be an useful parameter of antiinflammatory activity. As expected, all applied glucocorticoids inhibited TNFα stimulated II-1α production in a dose-dependent manner within 24 h. Effects were already seen at 10^{-9} M (data not shown). Most interestingly, however, there was a significant difference in the extent of the glucocorticoid mediated suppression. Whereas DM, PD, and BM at 10^{-8} M revealed minor effects only, BMV, PEC, and PC showed effective inhibition, reducing II-1α by 36 to 39% (Fig. 1) which suggests a high potency in the treatment of steroid-sensitive dermatoses. Remarkably, not only PC itself, but also its metabolites PEC and PD suppressed II-1α synthesis significantly (Fig. 1).

Due to the fact that double esters are especially subject to an intensive metabolism in keratinocytes (19), and II-1α concentrations were measured after 24 h, we also investigated the biotransformation of PC. In agreement with previous studies, cellular enzymes cleaved of the double ester at position 21. PEC was the main product during 12 h, after 24 h almost the entire PC was transformed. Considerable amounts of the final metabolite PD appeared after 6 h.

Regarding the rapid biodegradation of PC, it remained to consider whether II-1α suppression in keratinocytes was mediated by PC itself or its metabolites, predominantly PEC. Therefore, a RNAse protection assay was performed 3 h after stimulation by TNFα and subsequent addition of glucocorticoids (Fig. 2). The results show a strong antiinflammatory effect mediated by native PC. PEC appeared to be comparatively active.

Taking the results together, PC, PEC and BMV were identified as the most potent antiinflammatory agents, significantly more potent than DM, PD, and BM.

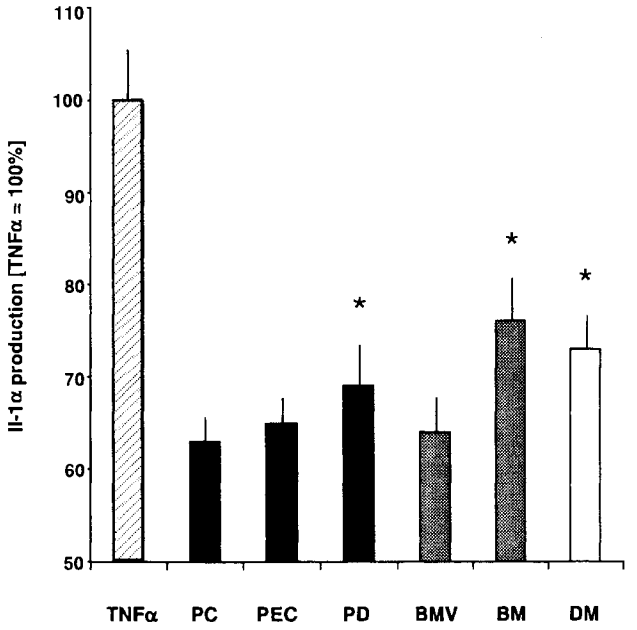

Fig. 1. Suppression of TNFα-induced II-1α production in keratinocytes (mean ± SD) by glucocorticoids (10^{-8} M). TNFα stimulated control = 100%. *: significantly different from PC.

Antiproliferative Effects of Topical Glucocorticoids on Fibroblasts

To evaluate the risk of topical glucocorticoid therapy for cutaneous atrophy we measured the influence of the test agents on fibroblast proliferation. In analogy to keratinocytes, cytokine production in fibroblasts was enhanced by TNFα. Subsequent glucocorticoid treatment induced a concentration dependent inhibition of II-1α production. There was a strong correlation

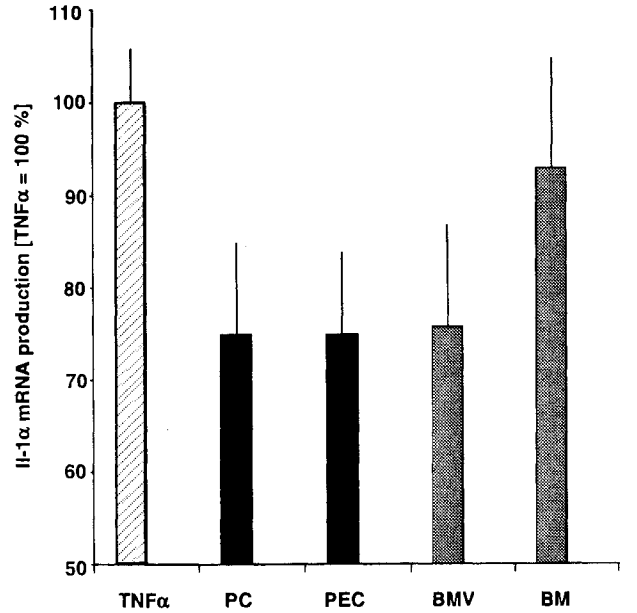

Fig. 2. Suppression of TNFα-induced II-1α mRNA in keratinocytes (mean ± SD) by glucocorticoids (10^{-8} M). TNFα stimulated control = 100%.

in the extent of II-1α (Fig. 3a) and II-6 (Fig. 3b) suppression ($r = 0.95$) after the treatment of 10^{-8} M glucocorticoids for 24 h.

In both assays, BMV appeared most potent, diminishing II-1α production by 38% and II-6 even by 76%, closely followed by DM and BM. PD was almost devoid of any influence. Most interestingly, the potent inhibitor of inflammatory II-1α synthesis in keratinocytes PC reduced II-1α production in fibroblasts by 15% and II-6 production by 13% only. Its main metabolite PEC, however, appeared almost as active as BMV, inhibiting II-1α by 25% and II-6 by 60%.

As shown previously (19), the biotransformation profile of both cells differ completely. In contrast to keratinocytes, PC is metabolized in fibroblasts by 1% per hour, suggesting minor esterase activity. Accordingly, the increase of the monoester PEC is rather low and concentrations of PD were below the limits of detection at any time. Therefore, the observed effects in fibroblasts can be ascribed to the native compounds.

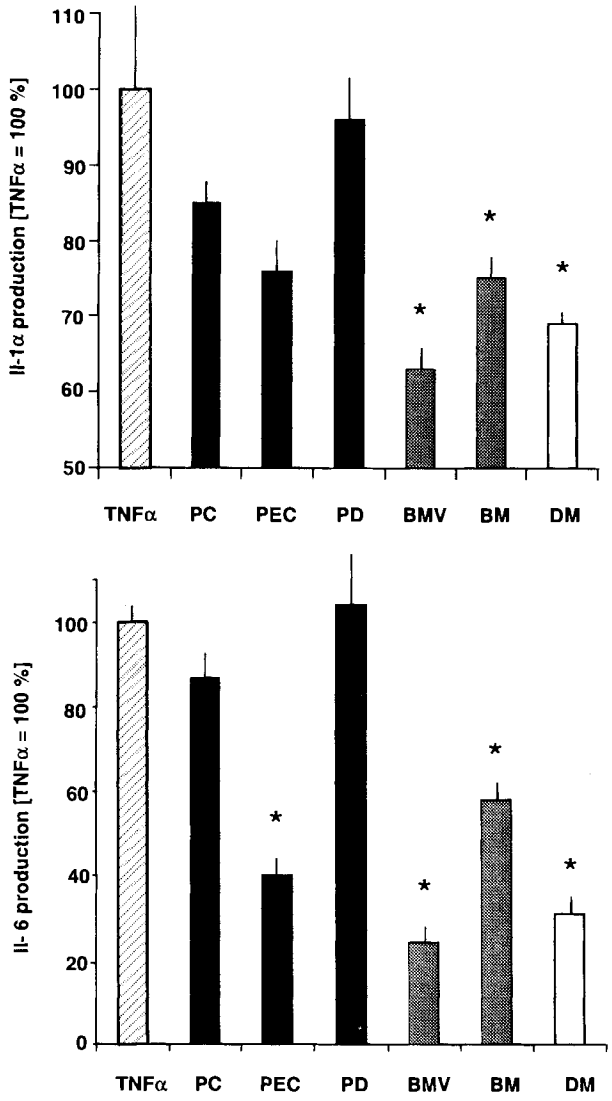

Fig. 3. Suppression of TNFα-induced II-α (3a) and II-6 (3b) production in fibroblasts (mean ± SD) by glucocorticoids (10^{-8} M). TNFα stimulated control = 100%. *: significantly different from PC.

To further elucidate antiproliferative effects, ^{3}H thymidine incorporation assays were performed. In agreement with our cytokine assay data, BMV appeared to be the most significantly antiproliferative agent, whereas PC reduced DNA synthesis even in 10^{-5} M to a minor extent only. PD, devoid of any significant reduction of ^{3}H thymidine incorporation, remained the least active substance in fibroblasts (Fig. 4). The same result was obtained with the MTT test, which confirmed antiproliferative influences observed by ^{3}H thymidine incorporation and cytokine assay (data not shown).

Taking the results together, PC has only minor antiproliferative effects in fibroblasts and its biodegradation to more toxic PEC occurs to negligible extent only. BMV, however, exerts a major influence on fibroblast proliferation, explaining dermal atrophy as observed in clinical trials.

Correlation of Benefit and Risk

To evaluate the benefit–risk ratio, we related the antiphlogistic effects of glucocorticoids in keratinocytes (benefit) with the antiproliferative actions in fibroblasts (risk). Substances revealing high antiphlogistic activity as derived from potent II-1α suppression in keratinocytes appear in the upper part of Figure 5. Agents with strong antiproliferative effects—obtainable by a major II-1α suppression in fibroblasts—are registered on the right hand side. A substance that may combine high benefit and low risk is to be found on the upper left of the figure. This holds true for PC and PD, the latter one, however, shows only discrete antiphlogistic potency in inflammatory skin disease.

DISCUSSION

In this paper we show, for the first time, the dissociation of beneficial and negative effects of topical glucocorticoids on the most important cells of the skin. Regarding the parameters used in the assay, efficient suppression of II-1α related inflam-

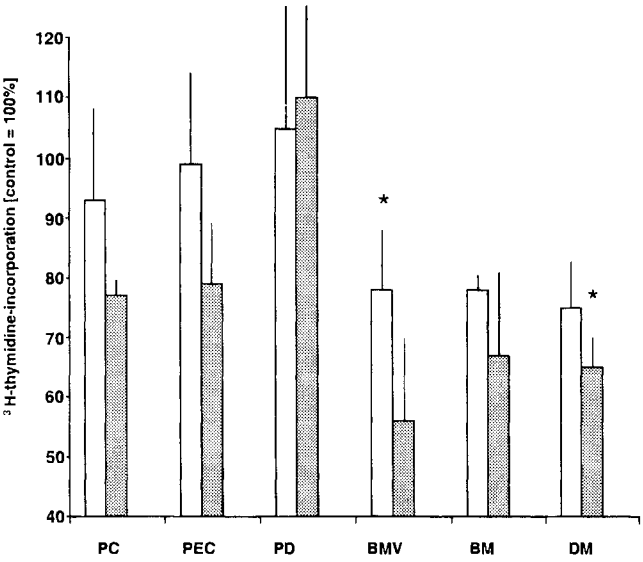

Fig. 4. Antiproliferative effects of glucocorticoids (10^{-6} M: open bars; 10^{-5} M: closed bars) in fibroblasts (mean ± SD) as measured by ^{3}H thymidine incorporation. Control = 100%. *: significantly different from PC.

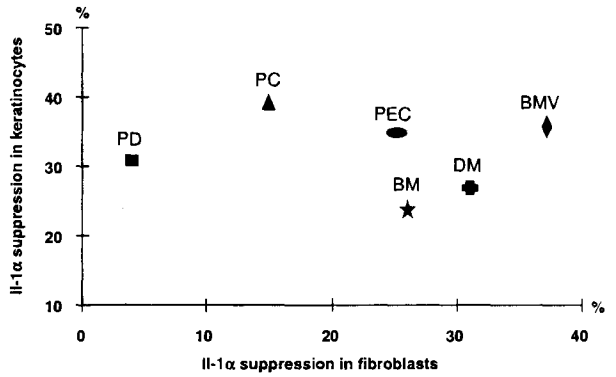

Fig. 5. Correlation of benefit (suppression of II-1α production in keratinocytes) and risk (suppression of II-1α production in fibroblasts) of 10^{-8} M glucocorticoids.

mation in keratinocytes holds for antiphlogistic effects (benefit), while suppression of II-1α and II-6 in fibroblasts stands for the unwanted, antiproliferative action (risk). Results were confirmed by proliferation assays as the ^{3}H thymidine incorporation and the MTT assay.

Evidence has accumulated about the genomic nature of glucocorticoid action, mediated by interacting with intracellular receptor proteins (20). These ligand activated transcription factors influence DNA transcription, resulting in the upregulation (e.g. lipocortin, neutral endopeptidase) or downregulation (cytokines, cyclooxygenase II) of mRNA and, subsequently, protein synthesis. As demonstrated recently, nuclear transcription factors like NFκB and inhibitory proteins as IκBα play a crucial role in the control of cytokine expression (9,10,11). Glucocorticoids interfere with the signal transduction either by enhancing inhibitory Iκ-Bα production which inactivates NFκB by complex formation (9,10) or by interacting with NFκB directly (11). Showing a reduced production of interleukin mRNA, our results also relate to a genomic mechanism. In additional experiments (data not shown) we found an even enhanced interleukin suppression (by about 20 %) by glucocorticoid preincubation 3 h before TNFα stimulation.

Interleukin suppression by topical glucocorticoids turned out to be a sensitive method to ascertain pharmacodynamic effects, as concentrations of 10^{-8} M were sufficient to attain significant reduction. ^{3}H thymidine incorporation, however, was not reduced until concentrations by 10^{-6} M were used. Especially in fibroblasts, low concentrations of PD and PC even induced cell proliferation. This is explained by the fact that fibroblast medium is devoid of hydrocortisone, which in concentrations up to 10^{-6} M serves as a growth factor. The difference of the active concentrations in the test systems used here appears suitable to estimate the therapeutic value of topical glucocorticoids. Manifest atrophogenic effects are not to be seen until higher concentrations are applied, as shown in the ^{3}H thymidine assay. The antiproliferative potential, however, becomes obvious by the low drug concentrations (10^{-8} M) used in the interleukin assay, which turns out as more sensitive.

The three conventional glucocorticoids tested, BMV, BM, and DM more or less exhibit classical action. They inhibit inflammatory response in keratinocytes and fibroblast proliferation in parallel. Especially BMV combines high benefit and high risk, as it exerts significant antiphlogistic effects in keratinocytes, but also suppresses II-1α and II-6 in fibroblasts most potently (Fig. 3). This strong antiproliferative property is proven once more by the ^{3}H thymidine incorporation (Fig.4). Its main metabolite, BM, combines low benefit and low risk, questioning its further value in dermatotherapy. DM did not show convincing antiinflammatory potency in the interleukin assay, but provided potent antiproliferative characteristics in the interleukin assay as well as in the ^{3}H thymidine incorporation study.

Prednicarbate induces potent antiinflammatory effects in keratinocytes corresponding to BMV activity, which is well within accordance with clinical data (5,6). The rapid metabolism of PC to PEC (19) does not contradict a substance specific antiphlogistic potency, as the RNAse protection assay proved native PC to be as active as BMV. Furthermore, PEC from PC metabolism contributes to the antiphlogistic effect, thus biodegradation in keratinocytes is less important for antiphlogistic means. The final metabolite PD exerts minor antiphlogistic activity. Thus at first the therapeutic effect depends on the most potent, native PC. After some hours, the effects are mainly due to PEC and in the end may result from the metabolite of lowest activity, PD.

In fibroblasts, native PC reveals minor antiproliferative activity as reflected by non-significant suppression of II-1α synthesis and by the weak influence on II-6 synthesis and cell proliferation. In contrast to PC, PEC exerts strong antiproliferative effects. PC, however, is metabolized in fibroblasts by 1% per h only, and PEC appears to a negligible extent (19). The present study does not exclude that in human PEC produced in keratinocytes may reach the dermis by diffusion to the deeper layers of the skin and may induce antiproliferative effects. Since, however, skin atrophy is not seen with prednicarbate cream (3) one may speculate on minor relevance of PEC diffusion. Though cellular uptake and penetration through the stratum corneum have been investigated in principle (20,21), detailed information of the tested drugs about the penetration and duration of stay within the skin are still lacking.

The question arises, how the different pharmacodynamic properties of topical glucocorticoids despite the identical mode of action can be explained. With respect to PC, affinity to the glucocorticoid receptor—obtained from lung tissue—is about the same as PD, whereas with PEC it is more than 10fold higher (22). A hypothetical explanation consists of different glucocorticoid receptor subtypes in keratinocytes and fibroblasts. The close correlation of the influence on II-1α and II-6 in fibroblasts with all glucocorticoids tested ($r = 0.95$) and the clear separation of effects on interleukin synthesis in keratinocytes versus fibroblasts hints at glucocorticoid receptor subtypes with different affinities for the drugs tested here. Recently, α and β subtypes of this receptor have been described (20). For the evaluation of this hypothesis, receptor binding studies have to be performed using keratinocytes and fibroblasts for receptor isolation.

In conclusion, the improved benefit–risk ratio of PC results from specific drug metabolism and selective influence on cytokine production in keratinocytes. Though particular therapeutic indications may need separate investigations (24), the presented in vitro assays appear suitable to predict the benefit-risk ratio of topical glucocorticoids during early drug development and will thus improve the finding of potential new derivatives of this class of agents without the need to perform animal experiments.

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