Acta Derm Venereol (Stockh) 1994; 74: 257–259

Potentializing Effect of Ketoconazole on Cyclosporin A-induced Inhibition of Keratinocyte DNA Synthesis

CAROLE AMSELLEM[1], MAREK HAFTEK[1], JEAN THIVOLET[1], PIET DE DONCKER[2] and DANIEL SCHMITT[1]

[1]INSERM U 346 affiliée CNRS, Department of Dermatology, E. Herriot Hospital, Lyon, France and [2]Janssen Research Foundation, Beerse, Belgium

Keratinocyte growth in vitro and DNA synthesis by epidermal cells in vivo are inhibited by therapeutic doses of cyclosporin A (CsA). This effect may be potentialized by topical treatment with ketoconazole, since this drug has been shown to inhibit CsA metabolism. Normal human skin grafts on nude mice receiving intraperitoneal injections of CsA were treated with ketoconazole cream or its placebo for 3 weeks. The keratinocyte DNA synthesis rate was evaluated through the rates of bromodeoxyuridine (BrdU) incorporation, and the trough blood levels of CsA were checked at the end of the experiment. Counting of the BrdU-labelled nuclei in human tissue sections confirmed a dose-dependent inhibition of BrdU incorporation by keratinocytes exposed to CsA. This CsA-induced inhibition was further increased in the animals treated with ketoconazole cream. This effect was best seen in the groups treated with the low-to-medium doses of CsA (12.5 and 25 mg/kg/day). However, the simultaneous increase in the circulating CsA levels was also observed in these animals. Based on our results, we speculate that the potentializing effect of ketoconazole on CsA-induced inhibition of keratinocyte DNA synthesis is systemic rather than local. ***Key words: human skin grafts; BrdU; keratinocyte proliferation.***

(Accepted December 20, 1993.)

Acta Derm Venereol (Stockh) 1994; 74: 257–259.

C. Amsellem, INSERM U 346, Pav. R, Hôp. E. Herriot, F-69437 Lyon cedex 03, France.

Cyclosporin A (CsA) a potent immunosuppressive drug of fungal origin, induces growth inhibition of normal (1–3) and pathological (4, 5) epidermal keratinocytes in vitro. It has been shown that CsA also exerts an antiproliferative effect in vivo on normal human skin xenografted onto nude mice (6). However, topical treatment with CsA does not result in clinical improvement of hyperproliferative psoriatic lesions (7), even when intralesional application of CsA is successful (8, 9).

Orally administered CsA is primarily metabolized in the human liver by the microsomal mono-oxygenase/cytochrome P-450 enzymatic system (10). Cytochrome P-450 activity has also been found in the epidermis of neonatal rats (11) and in the dermis of adult hairless mice (12). It is already known that ketoconazole, an antifungal drug, is a potent inhibitor of the cytochrome P-450 complex, and its administration may result in a decreased cellular metabolism (13).

The aim of this work was to study whether topical ketoconazole would potentialize the direct effect of CsA on keratinocyte DNA synthesis in vivo. To verify this hypothesis, we grafted normal human skin onto athymic mice and evaluated the rates of bromodeoxyuridine (BrdU) incorporation in animals treated systemically with various doses of CsA and receiving, in a "blind" manner, topically applied 2% ketoconazole cream or its placebo.

MATERIAL AND METHODS

Human skin grafts

Six-week-old congenitally athymic female nude mice (Swiss nu/nu, Iffa Credo, Les Oncins, France) were used in this study. Normal human skin from abdomen plastic surgery was keratotomized at a depth of 0.3 mm, and normal human skin xenografts (NHSX) of 1 cm^2 were prepared (14, 15). Briefly, after induction of pentobarbital anesthesia, human skin was placed dermal side down on graft beds prepared by excision of full-thickness mouse skin at the upper dorsal region of each animal. The grafts were fixed with transpore surgical tape and further dressed with gauze and self-adhesive tape.

Ten days after grafting, the dressings were removed, and the 56 mice bearing healthy-looking NHSX were randomly divided into eight groups of 7 animals. The mice were kept separately, one per cage, throughout the experiment.

CsA and ketoconazole treatment

Starting 11 days after grafting and over a 3-week period, the mice were treated intraperitoneally with three different daily doses of injectable CsA solution – Sandimmune (12.5, 25, and 50 mg/kg b.w.) – or with the equivalent volume (0.1 ml) of its placebo – Cremophore (generous gifts of Sandoz Laboratories, France), two groups per dose. Out of each pair, one group received topical applications of 2% ketoconazole cream, the other its placebo (both prepared and coded by Janssen Laboratories, Beerse, Belgium). The creams were lightly rubbed into grafts twice daily throughout the experiment.

BrdU incorporation and immunolabelling

Intraperitoneal injections of BrdU (100 mg/kg in physiological saline, Sigma, St Louis, MO, USA) were administered in all eight groups three times a day during the last 4 days of the experiment. On day 31, the mice were killed and their blood was collected for measurement of CsA trough levels by a radioimmunologic assay (Sandimmun-kit, CIS bio international, Gif-sur-Yvette, France). NHSX were then excised and saved for immunohistochemical studies. Each graft was fixed in a Baker's solution and paraffin-embedded. Anti-BrdU labelling and counting of the labelled nuclei on dewaxed tissue sections were performed as described (6, 16). After a 15-min digestion with 4N HCL at 37°C and a 5-min wash in 0.1 M borax buffer (pH 8.5), the sections were labelled overnight at +4°C with the anti-BrdU monoclonal antibody diluted 1:10 (Becton-Dickinson, San Francisco, CA, USA). The BrdU-positive cell nuclei were revealed using the streptavidin-biotin-alkaline phosphatase technique (LSAB-Kit, Dako, Glostrup, Denmark). The sections, counterstained with hemalum, were analysed under a microscope equipped with a semiautomatic quantitation device (Mini-Mop, Kontron, Germany).

The results, expressed per 1 mm^2 of epidermal section area, were compared using Mann-Whitney's U-test.

Involucrin staining

Confirmation of the human tissue survival by the specific staining with an anti-human involucrin polyclonal antibody (Biosys, Compiègne,

  ISSN 0001-5555

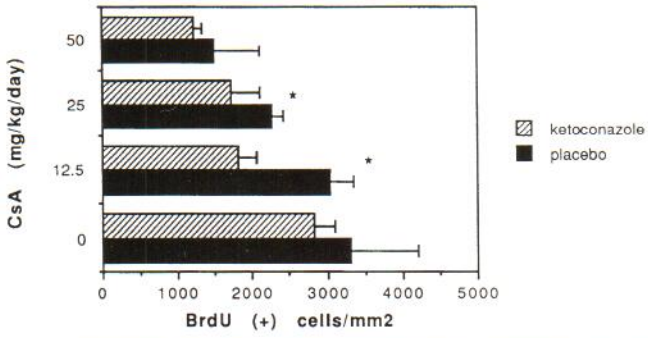

Fig. 1. Number of BrdU-positive nuclei per 1 mm² of epidermal section area of normal human skin xenografts treated or not treated with intraperitoneal CsA and topical ketoconazole. * = difference statistically significant (ketoconazole versus placebo, $p < 0.01$, Mann-Whitney U-test).

France) was performed on dewaxed tissue sections using the streptavidin-biotin-alkaline phosphatase technique (LSAB-Kit).

RESULTS

The grafted skin was well accepted in all the animals. The anti-involucrin stainings performed at the end of the experiment confirmed the human origin of the samples used for evaluation.

The results of the topical treatment (ketoconazole versus placebo) were read in a blind manner and analysed after decoding of the cream tubes by Janssen.

The quantitative results of the BrdU (+) cell counting are shown in Fig. 1. The CsA-induced decrease of the BrdU (+) cell number was more pronounced in the groups of animals receiving simultaneously topical ketoconazole (significant differences, $p < 0.01$, in groups treated with 12.5 and 25 mg/kg/day of CsA). The ketoconazole cream alone was unable to produce any significant modification of BrdU incorporation in grafted human keratinocytes. In all the groups, the number of BrdU (+) cells correlated well with the CsA dosage applied (inverse relationship).

CsA trough blood levels in mice treated intraperitoneally with 0, 12.5, 25, and 50 mg CsA/kg b.w./day in the absence of topical ketoconazole were < 25, 66.7 ± 11.7, 175.6 ± 74.9, and 736.8 ± 330.3 ng/ml (mean ± SD), respectively (Fig. 2). When the combined CsA-ketoconazole treatment was applied, these levels were slightly increased, the difference reaching statistical significance only in the group receiving 12.5 mg CsA/kg b.w./day ($p < 0.01$).

DISCUSSION

The results of our study indicate the presence of the potentializing effect of ketoconazole on CsA-induced inhibition of keratinocyte DNA synthesis.

This effect was best visualized in the groups treated with relatively mild doses of CsA. The lack of the significant additional DNA synthesis inhibition in the group of mice treated with 50 mg CsA/kg/day may be explained by the inhibitory effect close to maximum which is exerted by this high dose of CsA itself. The CsA dose-dependent influence on keratinocyte proliferation was related to the trough CsA blood levels observed in the animals, which confirms our previous results (3, 16). The additional decrease of BrdU incorporation occurring in animals receiving topical ketoconazole could also be related to the increased circulating CsA levels, even though the significant increase of the latter value concerned only the group receiving 12.5 mg CsA/kg b.w.

Thus, the problem which arises from this study is whether the topical application of ketoconazole cream may increase the level of circulating CsA in our experimental model. It is rather unlikely that the cutaneous metabolism of CsA in the ketoconazole-treated grafts could represent a sufficiently large fraction of the overall drug inactivation process to result in the systemic modifications of the CsA levels. On the other hand, previous studies addressing themselves to the question of transcutaneous penetration of the ketoconazole cream into human skin both in vivo (17, 18) and ex vivo (19, 20) proved that the active molecule was unable to pass into the circulation. Although the localization of our grafts (next to the neck) prevented licking of the treated skin, it is not impossible that mice scraped off and then ate some of the topically applied compound. There have been numerous reports showing that a combination of cyclosporin and ketoconazole given orally to rats (21, 22) and to human bone marrow (23) or kidney transplant recipients (24, 25) results in increased trough blood levels of CsA. Therefore, we speculate that the potentializing effect of ketoconazole on CsA-induced inhibition of keratinocyte DNA synthesis demonstrated in our study is systemic rather than local and may be due to the ingestion of ketoconazole cream by some of the mice. However, topically applied azole antifungals (e.g. ketoconazole) can inhibit cytochrome P-450 activity in the epidermis, as demonstrated by a decreased retinoic acid metabolism in rat skin microsomes (26). In this context, we cannot completely exclude the possibility that the enhancement of antiproliferative effects of CsA observed in our experiment was, at least partially, due to such a local ketoconazole action, and not exclusively related to the increase of circulating CsA levels. Although our study shows the combined action of the two drugs on keratinocyte proliferation, an attractive possibility that topically applied ketoconazole, which would remain confined to the lesional epidermis, and thus locally potentialize antiproliferative effects of CsA without raising its blood levels, could not be demonstrated.

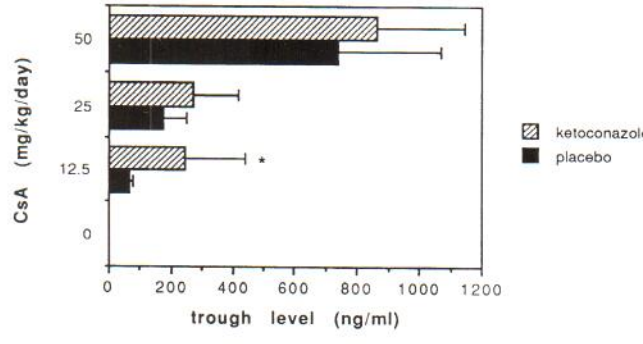

Fig. 2. Trough blood levels of CsA in mice treated or not treated with ketoconazole. * = statistically significant difference ($p < 0.01$) between groups receiving ketoconazole cream and its placebo (Mann-Whitney U-test).

ACKNOWLEDGEMENTS

This work was supported by the Janssen Research Foundation, Beerse, Belgium.

We thank Mr J. Carew for reviewing the manuscript, J. Croibier and J. Soum for their technical assistance, and Pr Ch. Bizollon for RIA measurements.

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