Reduced Expression of Cyclins and Proliferative Indices on the Psoriatic Epidermis after 12 Weeks of Oral Cyclosporin Therapy

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Cell growth characterized by cell cycle progression is regulated by cyclin-dependent kinase (CDK). CDKs are activated by binding cyclins such as cyclin A, cyclin B, cyclin D1, and cyclin E. Proliferative indices such as Ki-67 and proliferative cell nuclear antigen (PCNA) are known to be correlated with the mitotic index and were reported to have increased in the lesional psoriatic skin in previous reports. In this study, we investigated the expression of cyclins and proliferative indices (cyclin A, cyclin B, cyclin D1, cyclin E, Ki-67, and PCNA) in the psoriatic epidermis before and after cyclosporin therapy (3 mg/kg/day × 12 wks). Cyclin A, Ki-67, and PCNA were 1+ to 2+ positive before treatment but showed positive staining in only a few cells after treatment. Cyclin B and cyclin E were also moderate-to-strongly positive before treatment and became only weakly positive after treatment. Cyclin D1 was expressed only in a few cells and was negative after treatment. Taken together, cyclosporin may have an anti-proliferative effect on keratinocytes which was demonstrated by reduction of the proliferative indices such as Ki-67 and PCNA. The mechanism of the anti-proliferative effect may be through the inhibition of the cell cycle progression. Cyclin A, cyclin B and cyclin E are amongst the targeted cell cycle modulators, whereas cyclin D1 seems to be less induced in the lesional psoriatic epidermis, both before and after cyclosporin therapy.
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Key Words: Psoriasis, Cyclins, Cyclosporin

INTRODUCTION

There has been an increasing interest in the role of cell cycle related proteins such as cyclins, cyclin dependent kinases (CDKs), and cyclin dependent kinase inhibitors (CDKI) in the pathogenesis of psoriasis[1-3]. CDKs are activated by binding cyclins such as cyclin A, cyclin B, cyclin D1, and cyclin E. Proliferative indices such as Ki-67 and proliferative cell nuclear antigen (PCNA) are known to be correlated with the mitotic index[4-8] and were reported to have increased in the lesional psoriatic skin in previous reports[9,10].

To evaluate the hypothesis that the main mechanism of the anti-proliferative effect of cyclosporin on the psoriatic keratinocyte may be via modulation of the cell cycle-related proteins, we investigated the immunohistochemical expression and confocal laser microscopic localization of cyclins and proliferative indices in the psoriatic epidermis, before and after cyclosporin therapy.

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MATERIALS AND METHODS

Materials

Eight patients (5 females, 3 males, mean age 56.3 years) with severe chronic plaque-type psoriasis were included in our study. As a control group, 8 normal volunteers were selected. All patients underwent 12 weeks of oral cyclosporin (dose of 3 mg/kg/day) therapy, without any topicals.

Methods

Skin biopsy

Skin biopsy specimens were obtained from all patients with a punch biopsy from a psoriatic plaque, before treatment and at the end of 12 weeks of therapy. Eight normal skin samples of healthy volunteers were also obtained for comparison.

Formalin-fixed skin fragments were embedded in paraffin and processed for immunohistochemistry. For confocal microscopy, each punch biopsy fragment before treatment and at the end of 12 weeks of therapy was maintained in fixative containing paraformaldehyde and picric acid for 3 hours at 4°C and then rinsed in 15% sucrose and 0.15 mM sodium azide phosphate-buffered saline (PBS) overnight at 4°C. The samples were embedded in ornithine carbamyl transferase compound (Tissue-Tek OCT Compound, Sakura Fine Technical Co Ltd, Tokyo, Japan) and stored at -80°C.

Immunohistochemistry

For immunohistochemistry, 4μm-thick sections were deparaffinized in xylene three times, each for 10 minutes, hydrated, autoclaved in buffered citrate for 10 minutes and cooled at room temperature. Diluted primary antibodies shown in Table 1 were then applied. The slides were washed and incubated with secondary antibodies (biotinylated anti-rabbit and anti-mouse immunoglobulin), placed in phosphate-buffered saline, and incubated with diluted peroxidase conjugated streptavidin for 15 minutes. Finally, AEC (3-amino-9ethyl-carbazole; Dako, Carpinteria, CA, USA) was used as a chromogen. For the interpretation of the results, percentages of cyclin A-, cyclin B-, cyclin D1-, cyclin E-, Ki-67- and PCNA- positive epidermal cell nuclei were evaluated in 10 fields (at a magnification of × 400) and the percentage of positive cells and the intensity of staining were assessed (Table 1).

Table 1. Interpretation Criteria

% of positive cells		Intensity	
(-)	none	(-)	Negative
(±)	< 5%	(±)	Equivocal
(+)	5 - 10%	(+)	Weakly positive
(2+)	10 - 30%	(2+)	Moderately positive
(3+)	30 - 50%	(3+)	Strongly positive
(4+)	50% <		

Immunofluorescent staining for confocal microscopy

Transverse cryostat sections of 10-μm thickness were processed for immunofluorescent indirect double staining. The keratinocytes in the samples were labeled with a rabbit primary antibody above (Table 2) and a FITC-conjugated goat anti-mouse secondary antibody (1 : 400 dilution; Jackson Immunoresearch Laboratories, West Grove, PA, USA). Immunolabeling was performed in PBS containing 0.1% bovine serum albumin and 0.3% Triton X-100 for 1 hour. Sections were then rinsed, incubated with secondary antibodies for 1 hour in a dark at room temperature and mounted in aqueous antifading medium (Fluorescent mounting medium; Dako Corporation). Confocal microscopy was carried out using a Leica TCS-SPII laser scanning microscope with appropriate filters. Digitalized images were collected in successive frames of 1-μm serial optical sections and projected into a single image of a 50-μm section.

RESULTS

The results are summarized in Table 3 and are represented in the Fig. 1-6.

The cyclosporin therapy was well tolerated and there was no need for drug discontinuation in any of the patients. The clinical effect of cyclosporin treatment was satisfactory since it resulted in complete or near complete remission in all 8 patients. The initial mean PASI (psoriasis area and severity index) score of the 8 psoriatic patients was 31.3. At 12 weeks after cyclosporin treatment, the mean PASI score was 4.7 which was significantly lower in comparison with that of the baseline ($p < 0.05$, Wilcoxon signed rank test). Before therapy, typical psoriatic epidermal features with CD4+ CLA+ lymphocytes were seen histologically. After

Table 2. Primary Antibodies in this Immunohistochemical Study

Antibody	Dilution	Source
Cyclin D1	1 : 100	Novocastra, Newcastle, UK
Cyclin A	1 : 200	Novocastra, Newcastle, UK
Cyclin E	1 : 100	Novocastra, Newcastle, UK
Cyclin B	1 : 100	Novocastra, Newcastle, UK
Ki-67	1 : 100	Novocastra, Newcastle, UK
PCNA	1 : 200	Novocastra, Newcastle, UK

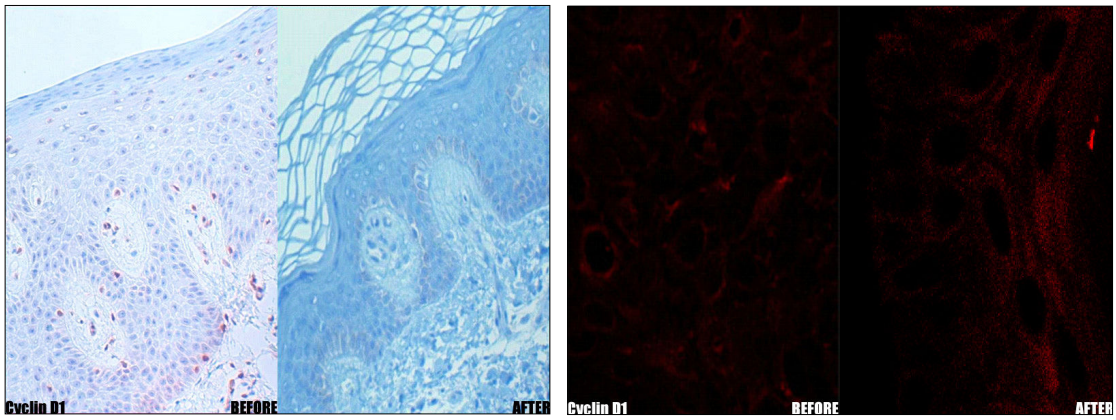

Fig. 1. Cyclin D1 expression was positive in few nuclei before treatment, whereas it was negative after treatment (immunohistochemical staining, original magnification × 200). Cyclin D1 expression in confocal microscopy is almost negative before and after treatment.

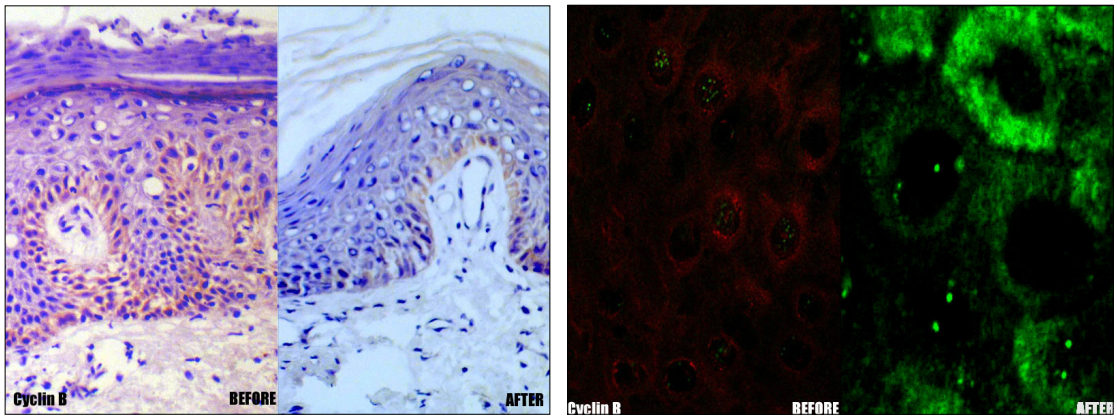

Fig. 2. Cyclin B expression showed a high positive rate of cytoplasm before treatment, whereas it became almost negative after treatment (immunohistochemical staining, original magnification × 200, inset: × 400). In confocal microscopy, it was strongly-positive before treatment and there was some positivity after treatment (only the green dot is interpreted as positive and the red is background staining).

Table 3. Summary of Immunohistochemial and Confocal Data

	Before treatment (N=8)		After treatment (N=8)	
		Confocal		Confocal
CyclinD1 positive rate	-(6/8) ~ ±(2/8)	-	-	-
Intensity	1+	-	-	-
CyclinE positive rate	2+(5/8) ~ 3+(3/8)	3+	1+(2/8) ~ 2+(6/8)	2+
Intensity	2+(5/8) ~ 3+(3/8)	2+	1+(7/8) ~ 2+(1/8)	1+(5/8) ~ 2+(3/8)
CyclinA positive rate	2+	2+(4/4) ~ 3+(4/4)	±	±
Intensity	3+	3+	2+	1+
CyclinB positive rate	2+	3+	±	2+1+
Intensity	2+	2+	1+	
Ki-67 positive rate	2+	N/A	±	N/A
Intensity	3+		2+	
PCNA positive rate	2+	N/A	±	N/A
Intensity	3+		2+	

N/A: confocal imaging was not performed

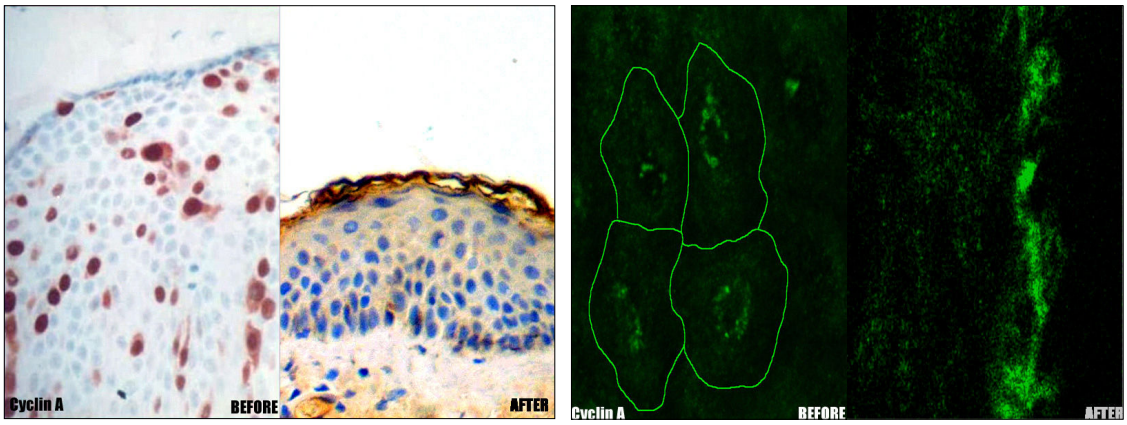

Fig. 3. Cyclin A expression was strongly-positive in the nuclei before treatment, but changed to weak positive in a few cells after treatment (immunohistochemical staining, original magnification × 400). Confocal microscopy confirmed the results.

therapy, the epidermal pattern normalized and inflammatory cells were only scarce (data not shown).

After 12 weeks of cyclosporin therapy, we observed the reduced expression of cyclins and cell proliferative indices on the psoriatic skin lesions (Fig. 1-6). Cyclin A, Ki-67, and PCNA were 1+ to 2+ positive before treatment but showed positive staining in a few cells after treatment. Before the 12 weeks treatment, nuclear positivity to cyclin A, Ki-67, and PCNA was observed in basal and parabasal areas, whereas in lesional skin after treatment and in healthy control, it was mainly shown in the basal layer when present (Fig. 2, 5, 6). Cyclin B and cyclin E were also moderately-to-strongly positive before treatment and became only weakly positive after treatment. While cyclin B and cyclin E were both intracytoplasmic and nuclear before therapy, only week diffuse intracytoplasmic positivity was seen in the lesional psoriasis after therapy (Fig. 2, 4). Cyclin D1 was expressed in only

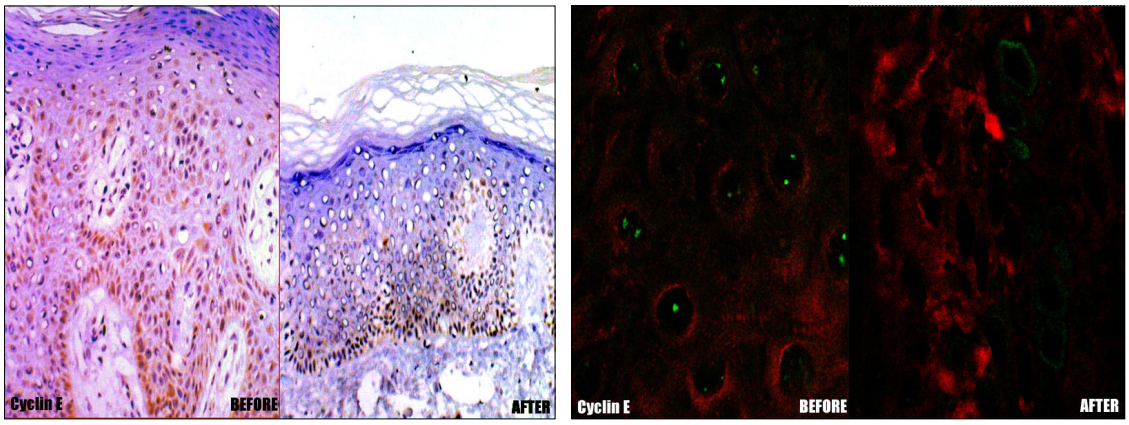

Fig. 4. Cyclin E expression showed a high positive rate of nuclei before treatment, whereas basal positivivty with less intensity after treatment (immunohistochemical staining, original magnification × 200). Confocal microscopy confirmed the results.

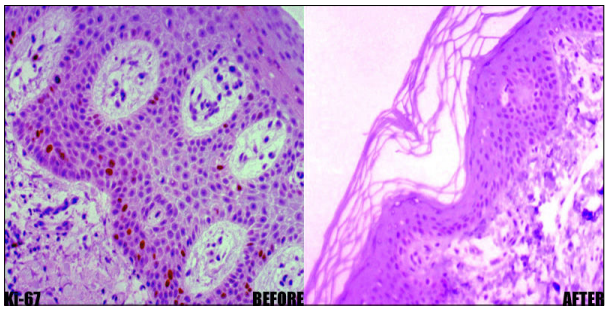

Fig. 5. Ki-67 expression was strongly-positive 2+ in nuclei before treatment, but changed to almost negative after treatment (immunohistochemical staining, original magnification × 200).

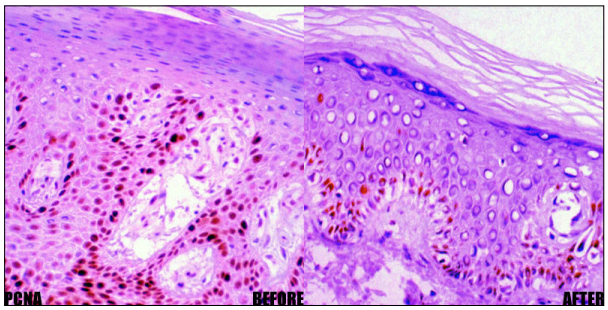

Fig. 6. PCNA expression was strongly-positive 2+ in nuclei before treatment, but decreased after treatment (immunohistochemical staining, original magnification × 200).

a few cells before treatment and was negative after treatment (Table 3). The positive staining rate of the lesional psoriasis after therapy was similar to that of normal skin. The results of a non-lesional skin biopsy in the psoriatic patients was not different from those of normal skin.

Confocal laser microscopic data (Fig. 1-4) were compatible with the results of immunohistochemistry. It confirmed the localization of each molecule in the nucleus or cytoplasm. Cyclin B and cyclin E were both intracytoplasmic and nuclear. Cyclin A was localized in the nucleus of the lesional sample. The positive rate of the lesional psoriasis after therapy was similar to that of normal skin. The results of a non-lesional skin biopsy in the psoriatic patients was not different from those of normal skin.

DISCUSSION

Psoriasis is an inflammatory dermatosis characterized by abnormal keratinocyte hyperproliferation. Lately, there is an increasing interest in cell cycle-related proteins to understand the pathogenesis of psoriasis[11].

Psoriasis has been classified as a T-cell-mediated, autoimmune disease[12-14]. An explosion of knowledge concerning immunological events in psoriasis and the clinical efficacy of immunologically directed therapies, such as cyclosporin, support this concept [13-15]. Some evidence suggests that cyclosporin has a direct effect on the proliferation and differentiation of psoriatic keratinocytes[15]. Some inhibitory effect on keratinocyte proliferation may be another anti-

psoriatic mechanism. In one previous report[16], however, the expression of epidermal growth factor (EGF) receptors on keratinocytes in all layers of the psoriatic epidermis persisted during cyclosporin treatment, despite resolution of the lesions.

Cell growth characterized by cell cycle progression is regulated by cyclin-dependent kinase (CDK)[1]. CDKs are activated by binding cyclins such as cyclin A, cyclin B, cyclin D1, and cyclin E[17]. Proliferative indices such as Ki-67 and proliferative cell nuclear antigen (PCNA) are known to be correlated with the mitotic index and were reported to be increased in the lesional psoriatic skin in previous reports[12,13].

Cyclins complexed with CDKs govern key transition in the cell cycle[1,2]. The expression of the cyclins seems to represent more specifically and differentiate the fraction of cycling cells[3]. CDKIs carry out a growth inhibitory function by blocking specific cyclin-CDK complexes[2-4]. Cyclins, CDKs, and CDKI are included in cell cycle related proteins. The expression of each molecule is not yet well characterized, although these proteins are considered to be important in hyperproliferative diseases, such as carcinomas[1,2,18-20].

Markers of cellular activity are of increasing interest in attempting to differentiate malignant from benign lesions[12,13,19]. Antibodies to PCNA are useful markers of the late G1 to S growth phase of the cellular proliferation cycle. Ki-67 is found to be positive in all phases of the cycle, while PCNA is expressed, although at very low levels, in non-cycling cells, accumulating in G1, peaking in the S phase but decreasing in G2/M phase[12,13,19]. Previous study indicated these indices were increased in psoriasis[13].

There have been only a few studies on the cyclin expressions in psoriasis *in vivo*[11,20,21]. In our study, the results were similar to previous studies[11,13,20,21]. Before therapy, cyclin A was expressed strongly-positive at a high rate, whereas cyclin D1 when positive, was expressed, usually not strongly in a small percentage of epidermal cell nuclei. Overexpression of cyclin D1 has been reported in many types of malignant tumors by immunohistochemical staining, but negative or only weak staining was observed in normal tissues and benign skin tumors[1,2]. These results imply that cyclin D1 expression is more related to a neoplastic condition rather than hyperproliferative skin states. Cyclin B links to CDK 1(p34) to form the so-called 'maturation/mitosis promoting factor'. This complex promotes mitosis until metaphase-anaphase. Therefore cyclin B expression reflects these phases of the mitotic process. In our study, its expression showed moderate-to-strong positive before therapy. Cyclin E also was overexpressed in psoriatic epidermis. Proliferative indices showed increased expression, paralleling with cyclin expression, in accordance with previous studies[11].

After 12 weeks of cyclosporin therapy, expression of cyclins and proliferative indices were reduced, with the decrease of cell proliferation and the normalization of epidermal pattern and thickness. Its primary mechanism of action was suppression of T cell proliferation and activation by the inhibition of various cytokine production by activated T cells. However, some studies have reported other mechanisms of action in cyclosporine[3,15]. *In vitro*, a direct effect of cyclosporin on epidermal cell proliferation was observed[4]. Cyclosporin binds to a protein, cyclophilin, blocking the cell cycle in the G0/G1 phase, although its mechanism is not completely known[5-7]. In another study, cyclosporin was reported to induce cell cycle arrest by increase of p21 and p53 in renal epithelial cells[8]. Furthermore, modification of cell adhesion to the matrix and of the basal membrane may produce epidermal proliferation, as observed in some studies[9,10,22]. Until now, which of these results are due to a direct effect of cyclosporin on the keratinocyte is not certain[23-29]. And the anti-proliferative effect of cyclosporin measured by proliferative indices is restricted only when a high dose of cyclosporine is applied *in vivo*, even though it shows an anti-proliferative effect *in vitro*[4,7,8]. So it might be possible that something different mechanism from that observed in other studies could be playing an anti-proliferative effect in this study.

The results of our study suggest the possibility that cyclosporin may have an anti-psoriatic mechanism through the modification of cell cycle related proteins, especially in relation to cyclin A, B, E rather than D1.

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