Rapamycin (sirolimus) inhibits proliferating cell nuclear antigen expression and blocks cell cycle in the G1 phase in human keratinocyte stem cells.

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*J Clin Invest.* 1997; 99(9):2094-2099. [https://doi.org/10.1172/JCI119382](https://doi.org/10.1172/JCI119382).

Because the immunosuppressant rapamycin (sirolimus) blocks T cell proliferation in G1 phase, it has been proposed as a potential treatment for psoriasis, a skin disease characterized by T cell activation and keratinocyte stem cell hyperproliferation. To determine another potentially important mechanism through which rapamycin can act as an antipsoriatic agent, we tested its direct effect on keratinocyte stem cell proliferation in vitro as well as in vivo. In vivo cell cycle quiescent (G0 phase) stem cell keratinocytes in primary culture sequentially express de novo cyclin D1 and proliferating cell nuclear antigen (PCNA), prior to S phase entry, and upregulate beta1 integrin. Rapamycin inhibited the growth of keratinocytes that were leaving quiescence as well as those already in cell cycle without affecting cell viability. Although beta1 integrin(bright) expression was not affected, the number of beta1 integrin(bright) cells entering S/G2/M was significantly lowered by rapamycin. Cells treated with rapamycin exhibited decreased PCNA expression while cyclin D1 expression, which precedes PCNA expression in the cell cycle, was not affected. We found similar effects on stem cell keratinocytes in patients with psoriasis treated systemically with rapamycin. Because PCNA is required for cell cycle progression from G1 to S phase, our data indicate that inhibition of PCNA protein synthesis may be an important regulatory element in the ability of rapamycin to exert a G1 block.

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Rapamycin (sirolimus) Inhibits Proliferating Cell Nuclear Antigen Expression and Blocks Cell Cycle in the G₁ Phase in Human Keratinocyte Stem Cells

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Abstract

Because the immunosuppressant rapamycin (sirolimus) blocks T cell proliferation in G₁ phase, it has been proposed as a potential treatment for psoriasis, a skin disease characterized by T cell activation and keratinocyte stem cell hyperproliferation. To determine another potentially important mechanism through which rapamycin can act as an antipsoriatic agent, we tested its direct effect on keratinocyte stem cell proliferation in vitro as well as in vivo. In vivo cell cycle quiescent (G₀ phase) stem cell keratinocytes in primary culture sequentially express de novo cyclin D1 and proliferating cell nuclear antigen (PCNA), prior to S phase entry, and upregulate β₁ integrin. Rapamycin inhibited the growth of keratinocytes that were leaving quiescence as well as those already in cell cycle without affecting cell viability. Although β₁ integrin bright expression was not affected, the number of β₁ integrin bright cells entering S/G₁/M was significantly lowered by rapamycin. Cells treated with rapamycin exhibited decreased PCNA expression while cyclin D1 expression, which precedes PCNA expression in the cell cycle, was not affected. We found similar effects on stem cell keratinocytes in patients with psoriasis treated systemically with rapamycin. Because PCNA is required for cell cycle progression from G₁ to S phase, our data indicate that inhibition of PCNA protein synthesis may be an important regulatory element in the ability of rapamycin to exert a G₁ block. (J. Clin. Invest. 1997. 99:2094–2099.) Key words: keratinocytes • PCNA • psoriasis

Introduction

In this study, we examined the effect of rapamycin (sirolimus) on in vitro primary epidermal cell cultures. In primary culture, keratinocyte stem cells acquire high levels of β₁ integrin, exit G₀, and enter the proliferative phase of the cell cycle (1, 2). To identify the point in cell cycle at which rapamycin acts, we used flow cytometric analysis using antibodies to mark stem cell activation (β₁ integrin), G₁ points in cell cycle (proliferating cell nuclear antigen (PCNA) and cyclin D1), as well as DNA content to mark S and G₂/M progression from G₀/G₁.

PCNA is first expressed in mid-G₁ (3). PCNA expression peaks in S phase but continues to be weakly expressed in G₂ and M phases of the cell cycle (3). Not only is it involved in DNA replication (4, 5) and repair (6), but it also plays a role in cell cycle regulation (7, 8). D-type cyclins are important for progression from the G₂/M to S phases of cell cycle (9–11). They are cell-type specific; cyclin D2 is thought to be the major D-type cyclin expressed in keratinocyte cells (12). However, we have recently shown that keratinocytes express cyclin D1 when they traverse from cell cycle quiescence (G₀) into cell cycle prior to G₁ PCNA expression (13).

In vivo epidermal growth is regulated by the degree to which stem cells in basal epidermis remain in the quiescent (G₀) or proliferative (G₂/S/G₂/M) phase of cell cycle. This process is aberrant in psoriasis. Epidermal hyperproliferation in psoriasis is characterized by a markedly increased percentage of normally quiescent basal (stem) keratinocytes in the proliferative phases of cell cycle (14). T lymphocytes infiltrating psoriatic lesions may play a role in the induction and maintenance of keratinocyte hyperproliferation in the disease (15). Cyclosporine and FK-506 (tacrolimus), macrocyclic immunosuppressants that block T cell activation, have been used as treatments for psoriasis but their use is often times limited because of side effects such as nephrotoxicity and hypertension.

Rapamycin is a macrocyclic immunosuppressant with demonstrated ability to block T cell proliferation via a G₁ cell cycle blockade (16). Rapamycin does not block the cyclinoprotein and FK-506–sensitive calcium-dependent pathway that is critical for T cell receptor-mediated signal transduction, but it does inhibit the S6 kinase pathway used not only by T cells but also by other cell types for cell cycle progression (17–19). Whereas cyclosporine and FK-506 exert minimal effects on keratinocyte proliferation at dosages achievable in vivo (20), rapamycin, because of its distinct cell cycle signaling target of action, may inhibit keratinocyte growth in vitro (21). If rapamycin indeed blocks keratinocyte proliferation as well as T cell activation, it could be a treatment with rapid effects in psoriasis. Data presented here indicate that rapamycin does not affect stem cell β₁ integrin activation. Rather, rapamycin acts af-

J. Clin. Invest. © The American Society for Clinical Investigation, Inc. 0021-9738/97/05/2094/06 $2.00 Volume 99, Number 9, May 1997, 2094–2099

1. Abbreviations used in this paper: EtOH, ethanol; K1/K10, keratin 1/keratin 10; PCNA, proliferating cell nuclear antigen; PI, propidium iodide.
Rapamycin Inhibits Proliferating Cell Nuclear Antigen Expression

**Methods**

**Human subjects.** Keratome biopsies were taken from the buttock area of normal volunteers. As part of a phase I pharmacokinetic clinical trial, patients with psoriasis received 5 mg/m² per d oral rapamycin for 1 wk. Keratome biopsies were also taken from these patients before treatment (day 0), 2 d into treatment, and on the last day of treatment (day 7). Patients had not used systemic immunosuppressives for at least 4 wk and systemic retinoids, corticosteroids, or phototherapy for at least 3 wk before entry into the study. All procedures were approved by the Institutional Review Board of the University of Michigan Medical Center, and informed consent was obtained from each subject.

**Initiation of primary cultures.** Epidermal cell suspensions were prepared from keratomes of normal subjects as described previously (14). Cells were plated at a concentration of 1 × 10⁶ cells/ml into 6-well culture plates (2 ml/well) or into 25 cm² culture flasks (5 ml/flask) and cultured in keratinocyte basal media (KBM) (Keratinocyte-SFM medium without bovine pituitary extract and EGF; GIBCO Laboratories, Grand Island, NY) supplemented with 1% FBS. Cultures were treated with 20 nM rapamycin (Wyeth-Ayerst, Philadelphia, PA) dissolved in absolute ethanol (EtOH) or with absolute EtOH alone as negative control 2 h or 24 h after initiation of culture (short-term culture) or 72 h after initiation of culture (long-term culture). After 72 h of additional culturing following rapamycin or EtOH treatment, the adherent cells were treated with trypsin (0.025% trypsin + 0.01% EDTA) and collected. Cell viability was assessed with trypan blue (GIBCO Laboratories) to determine rapamycin cytotoxicity. Cells were then fixed in 70% EtOH and kept at −20°C at least overnight.

**Colony formation assay.** Epidermal cell cultures were prepared as described above. After culturing, cells were fixed in 1% formalin for 15 min on the culture plates at 4°C and stained with a solution of 50% Nile blue and 50% rhodamine B (both from Sigma Chemical Co., St. Louis, MO).

**Flow cytometric assay of keratinocyte subset proliferation.** The EtOH-fixed cells were stained with the following mAbs: anti–β1 integrin (4B4; Coulter Immunology, Hialeah, FL), anti–PCNA (PC10; Boehringer Mannheim Biochemicals, Indianapolis, IN), anti–cyclin D1 (PharMingen, San Diego, CA), anti–keratin 1/keratin 10 (K1/K10) (ICN Pharmaceuticals Inc., Costa Mesa, CA), and isotype controls (purified mouse IgG1 and IgG2a; Sigma Chemical Co.); then stained with the secondary antibodies goat anti–mouse IgG1 conjugated to FITC and IgG2a conjugated to PE (both from Boehringer Mannheim Biochemicals) and resuspended in propidium iodide (PI) with RNase A, both from Sigma Chemical Co., as in the method of Bata-Csorgo et al. (1) and Gong et al. (22). Samples were stored at 4°C and analyzed by flow cytometry within 24 h, described in detail elsewhere (14). Data were analyzed using Coulter Elite (Coulter Cytometry) and Modfit (Verity Software House, Inc., Topsham, ME) software.

**Statistical analysis.** Paired two-tailed Student’s t test was used to compare rapamycin-treated and control cultures.

**Results**

**Rapamycin inhibits establishment of keratinocyte primary cultures.** Normal fresh ex vivo epidermal cells were plated in KBM + 1% FBS. In order to capture potential rapamycin effects on cells leaving quiescence as well as cells already in cell cycle, we treated cultures with rapamycin 1 and 3 d after plating. At both time points, cell growth was inhibited by rapamycin treatment as demonstrated by reduced colony numbers and size (n = 8). Fig. 1 shows a representative experiment. At a concentration of 20 nM, rapamycin did not affect cell viability as determined by trypan blue staining of harvested cells (data not shown).

**Rapamycin does not affect β1 integrin upregulation.** To determine whether reduced culture establishment (by morphology) was because of an inhibition of the ability of stem cell keratinocytes to upregulate their β1 integrin expression, and thereby affect adhesion/signaling, we analyzed 3-d-old cultured keratinocytes that were exposed to rapamycin 2 or 24 h after plating using a flow cytometric assay described by Bata-Csorgo et al. (1). Keratinocyte stem cells will express high levels of β1 integrin (β1 integrinbright population) by about 72 h of culture but at 48 h after plating, a β1 integrinbright population is usually not yet evident.

Cultures stained with anti–β1 integrin FITC demonstrated that rapamycin treatment had no effect on the percentage of β1 integrinbright cells appearing in the culture by 72 h, as compared with the EtOH-treated control culture in four of four experiments. The average difference in β1 integrinbright cells between the treated and untreated cultures was 4±1% (Table I, compare columns 2 and 3).

![Figure 1](image-url)
Table I. Effect of Rapamycin on Cell Cycle of β1 Integrin<sup>bright</sup> Cells*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Rapamycin</th>
<th>Medium</th>
<th>Rapamycin</th>
<th>Medium</th>
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<tbody>
<tr>
<td>1</td>
<td>43.6</td>
<td>37.1</td>
<td>18.5</td>
<td>33.8</td>
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<tr>
<td>2</td>
<td>14.5</td>
<td>11.2</td>
<td>17.2</td>
<td>30.1</td>
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<td>17.1</td>
</tr>
<tr>
<td>4</td>
<td>53.3</td>
<td>57.8</td>
<td>24.2</td>
<td>52.5</td>
</tr>
</tbody>
</table>

*Normal adult epidermal cells were cultured in KBM + 1% FBS and treated with 20 nM rapamycin in EtOH or an equal volume of EtOH as negative control 2 h after plating. Adherent cells were harvested 72 h later, fixed in 70% EtOH and co-stained with anti-β1 integrin and PI for cell cycle analysis.

**PCNA expression is inhibited by rapamycin.** PCNA expression can be used as a marker of cell entry into G<sub>1</sub>. The PCNA mAb (clone PC10) used in this study labels both free PCNA and PCNA associated with replicating DNA. However, Bravo and McDonald-Bravo (4) have shown that only PCNA associated with DNA replication sites is detected after methanol fixation. This same study as well as others (5, 6) also show that, after Triton extraction, only the bound PCNA remains in cells. Therefore, as a result of fixation and extraction, the PCNA mAb is labeling only the PCNA associated with replicating DNA. However, 48 h in culture few cells, if any, express PCNA. By 72 h, however, proliferating cells (which are primarily stem cells and thus, β1 integrin<sup>bright</sup>) are expressing PCNA. In all short-term culture experiments (n = 4) rapamycin inhibited PCNA expression. The PCNA expression of the rapamycin-treated culture is only slightly higher than the nonspecific binding of the isotype control while the EtOH-treated culture has higher PCNA expression (Fig. 2). Both a PCNA<sup>bright</sup> and a PCNA<sup>low</sup> population are present in the EtOH-treated control culture (Fig. 2, solid thin line). By contrast, the rapamycin-treated culture contains only the PCNA<sup>low</sup> population (Fig. 2, dashed line). The reduction in PCNA expression is almost a log less, indicating a significant, but not complete, inhibition of PCNA expression. There was an average 48±6% reduction in the PCNA mean channel fluorescence of the rapamycin-treated cultures relative to the PCNA mean channel fluorescence of the EtOH control cultures.

**Cyclin D1 expression is not affected by rapamycin.** Cells express D-type cyclins in G<sub>1</sub> and the association of these cyclins with their cyclin-dependent kinases and PCNA suggests that they may be crucial for G<sub>1</sub> restriction point (start) regulation. Because adult keratinocytes express cyclin D1 and not cyclin D2 in their transition from G<sub>0</sub> to G<sub>1</sub>/S while already cycling keratinocytes express cyclin D2 (13), short-term primary cultures treated with rapamycin 2 h after plating were fixed and stained with anti-cyclin D1. Relative to isotype control (Fig. 3, solid dark line), the antibody-stained cells in both rapamycin-treated and EtOH-treated cultures exhibited the same cyclin D1 expression, as indicated by essentially overlapping histograms of cell count versus fluorescence intensity (Fig. 3, dashed and solid thin lines). The difference in cyclin D1 mean channel fluorescence was only 5±5% (n = 2). Both the rapamycin-treated and the EtOH-treated cultures contained positive cells stained with anti-cyclin D1 in equal numbers as indicated by similar numbers of cells with increased fluorescence intensity relative to the isotype control staining.

**Stem cell proliferation is inhibited by rapamycin.** Although PCNA expression was markedly reduced, it was not completely inhibited by rapamycin treatment. In the EtOH-treated control culture, there is a population with intermediate expression of PCNA (PCNA<sup>int</sup> population) which corresponds to the level of PCNA expression of the majority of rapamycin-treated cells still expressing PCNA (Fig. 2). To determine if this degree of PCNA reduction was associated with blocked progression into actual DNA synthesis (S/G<sub>2</sub>/M phase), short-term cultures were treated with rapamycin or EtOH and the adherent cells collected and fixed with 70% EtOH. The cells were then co-stained with anti-β1 integrin and the DNA stain PI. The relative size of the β1 integrin<sup>bright</sup> cell population (percent β1 integrin<sup>bright</sup> cells of all harvested epidermal cells) and the percent S/G<sub>2</sub>/M phase cells among the β1 integrin<sup>bright</sup> cells for each experiment are shown in Table I. Although the appearance of β1 integrin<sup>bright</sup> cells was not affected by rapamycin treatment (Table I, compare columns 2 and 3, P = 0.46), rapamycin did indeed suppress the number of β1 integrin<sup>bright</sup> cells undergoing DNA synthesis, based on cellular PI content (n = 4,

Figure 2. PCNA expression of rapamycin-treated and EtOH-treated short-term keratinocyte cultures. Normal adult epidermal cells were plated in KBM + 1% FBS and treated with rapamycin or control EtOH 2 h after plating. 72 h after plating, adherent cells were harvested and fixed in 70% EtOH, then stained with anti-PCNA PE. The one-parameter histogram shows PCNA expression (PE) of the epidermal cells along the x-axis. Non-specific fluorescence was detected by isotype staining (solid dark line). The culture treated with EtOH (solid thin line) had a distinct PCNA<sup>+</sup> population while the culture treated with rapamycin (dashed line) was only slightly brighter than the isotype control.
52 ± 5% decrease in S/G_{2/M} phase cells) (Table I, compare columns 4 and 5, \( P = 0.02 \)). Thus, the decreased PCNA expression because of rapamycin treatment is associated with a block in the ability of stem cells entering G_{1} (\( \beta 1 \) integrin_{bright} cyclin D_{1}^{+}) to transit into S phase. Other studies have suggested that cells will not enter S phase without a critical level of PCNA (8). Although the rapamycin-treated cells express an intermediate level of PCNA, these cells may lack this critical PCNA level and therefore be unable to enter S/G_{2/M}, as our data have shown.

**Rapamycin inhibits epidermal stem cell PCNA expression and proliferation in vivo.** To determine if these effects documented in primary ex vivo cultures are achievable in vivo, keratomes from patients with psoriasis who received rapamycin (5 mg/m\(^2\) per d) were examined for epidermal keratinocyte stem cell PCNA expression and cell cycle inhibition. Keratomes were taken at three time points: before treatment (day 0), two days into treatment, and on the final day of treatment (day 7). Epidermal cells were fixed with 70% EtOH and stained with anti-K1/K10, anti-PCNA, and PI (to quantify cellular DNA content). Over time, the PCNA mean channel fluorescence of the epidermal stem cell population (K1/K10\(^{-}\) cells) decreases with time (\( n = 2 \)) (Fig. 4). These results indicate that the PCNA expression in individual stem cells was inhibited by rapamycin treatment in vivo. The cell cycle analysis of the K1/K10\(^{-}\) population demonstrates a similar trend. In both subjects, the percent of cells in S/G_{2/M} decreased over time (Fig. 5). Our data show that rapamycin inhibits PCNA expression and stem cell proliferation not only in the in vitro keratinocyte cultures but in keratinocytes from patients with psoriasis as well.

**Discussion**

We have shown that rapamycin inhibits normal human stem cell keratinocyte proliferation by blocking cell cycle progression in G_{1} phase. Numerous studies have shown a similar effect on T cells. Thus, rapamycin may be a novel treatment for psoriasis, a skin disease with characteristic T cell infiltration and keratinocyte hyperproliferation.

The specific point in G_{1} at which keratinocyte stem cells were blocked was localized to be prior to PCNA expression but after cyclin D_{1} induction. Cyclin D_{1} expression precedes expression of PCNA as quiescent (G_{0} phase) stem cell keratinocytes progress into cell cycle (13). \( \beta 1 \) integrin upregulation, another characteristic change that keratinocytes undergo in the early in vitro culture as they progress from G_{0} into G_{1} (1, 2), was not affected by rapamycin. Both cyclin D_{1} and PCNA expression require de novo protein synthesis in these cells, unlike \( \beta 1 \) integrin_{bright} expression which is probably regulated through
changes in the glycosylation state of integrin subunits rather than new synthesis of integrin subunits (23).

The gene for the mid-G1, PCNA protein is regulated by the E2F transcription factor (24). It has been shown that, other than regulating DNA synthesis, PCNA may be important in cell cycle progression. New synthesis of PCNA is required before cells can enter S phase (25). Studies using antisense oligonucleotides to PCNA or its mRNA have demonstrated that these forms of treatment prevent cell entry into S phase (7, 8). The level of PCNA expression is highest at the G1/S transition, when cells are progressing in the cell cycle (25, 26).

Because PCNA expression is critical for cell cycle progression into S phase, the suppression of PCNA protein production may be the critical restricting mechanism of rapamycin’s ability to block keratinocyte proliferation.

Previous studies have shown that rapamycin inhibits p34^cdc2 kinase activity (27, 28), p34^cdc2 has been implicated in regulation of the G1/S phase transition in mammalian cells. p34^cdc2 may be involved in phosphorylation of the retinoblastoma gene product, p110^Rb, p110^Rb links the cell cycle with gene transcription by controlling the expression of genes necessary for progressing through cell cycle (29–31).

Rapamycin has been found to inhibit p110^Rb phosphorylation (32, 33). In its hypophosphorylated form, p110^Rb has been found to complex with the cellular transcription factor E2F (34, 35). Thus, rapamycin-induced hypophosphorylation of p110^Rb, because of the drug’s inhibition of p34^cd2 kinase activity, allows it to remain bound to E2F. In this complex form, E2F is inactive (35–37) and, as such, cannot transcribe the PCNA gene. Through this pathway, rapamycin may prevent expression of E2F-regulated proteins critical for G1 progression, such as PCNA.

Other studies have shown that rapamycin blocks phosphorylation of a specific ribosomal protein, S6, by inhibiting p70 S6 kinase activation (38–40). The actual function of phosphorylated S6 is unclear but it has been correlated with growth and increased protein synthesis in rat thymocytes (41).

Terada et al. (42) have shown that rapamycin inhibits up-regulation of ribosomal protein mRNA and protein in mitogen-activated primary T lymphocytes. Their study found that rapamycin did not affect the levels of nonribosomal proteins but did delay the synthesis of late G1 and S phase proteins (42). Protein synthesis is required throughout G1 in order for cells to progress into S phase (43).

Our work is the first to demonstrate that rapamycin inhibits the synthesis of PCNA, a cell cycle regulatory protein necessary for cells to traverse from G1 into S phase. The inhibition of PCNA synthesis by rapamycin can serve as an important tool in the investigation of cell cycle regulation. Our results also show that the epidermis, by harboring cell cycle quiescent (G0 phase) stem cells which uniquely establish in vitro keratinocyte colonies provides a useful system for investigating cell cycle regulation.

Acknowledgments

We thank Robin Gardner, Laura Van Goor, Kris Weber, and Rodney Weir for their technical assistance. We are grateful to Dr. Craig Hammerberg for generously providing invaluable advice.

This work was supported in part by grants from Wyeth-Ayerst Research, Sandoz Pharmaceuticals through the Dermatology Foundation, National Institutes of Health grant NIAMS Ro-1 (AR 41707-01), and the VA Medical Research Service.

References

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...tion and fluctuation in the level of the proliferating cell nuclear antigen and myc oncoproteins in synchronized neuroblastoma cells. Cancer Res. 53:1899–1905.


Cell. 65:1053–1061.


