Biochemical Pharmacology, Vol. 51, pp. 1315–1320, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00039-1

Antioncogene P53 and Mitogenic Cytokine Interleukin-8 Aberrantly Expressed in Psoriatic Skin Are Inversely Regulated by the Antipsoriatic Drug Tacrolimus (FK506)

Günter Michel,*§ *Hans Auer*,†,
Lajos Kemény,‡ *Alfred Böcking*† *and Thomas Ruzicka**

*Department of Dermatology and †Department of Cytopathology, University of Düsseldorf, Postf. 101007, D-40 001 Düsseldorf, Germany, and ‡Department of Dermatology, University of Szeged, Postf. 480 H-6721 Szeged, Hungary

ABSTRACT. Uncontrolled proliferation of epidermal cells is the most prominent characteristic of psoriasis. This widespread skin disease can be effectively treated with the microbial substance FK506, which acts by modulating gene expression. We, therefore, asked if the drug changes the expression of genes involved in growth regulation (the mitogenic cytokine interleukin-8 (IL-8) and p53, a negative cell cycle regulator) and signal transduction (protooncogenes c-ras, c-raf, and HER-2). Gene expression was monitored by semiquantitative mRNA-PCR and for p53 by immunocytochemistry in cultured primary keratinocytes (KC). In addition, p53 expression was analysed in skin biopsies of psoriatic patients. After 1–3 hr, IL-8 mRNA levels were dose-dependently decreased in tacrolimus (FK506)-treated cells. Protooncogene expression was not significantly altered. Interestingly, p53 transcription was clearly induced by FK506 treatment. This tendency could be verified on the protein level by immunocytochemistry. In contrast, p53 expression was decreased in lesional psoriatic as compared to normal skin, providing evidence that not only posttranslational modification of the p53 protein, but also transcriptional modulation of the p53 gene, are involved in pathological processes and pharmacological drug action in skin. Together with earlier results showing downmodulation for IL-8 receptor type A expression in cultured KC treated with FK506, these results suggest that both the mitogenic IL-8/IL-8R system and the cell cycle inhibitor p53 represent potential targets for the antipsoriatic action of the drug, whereas protooncogenes acting downstream in mitogenic signal transduction cascades are unaffected. The differential modulation of an entire set of genes provides evidence for the specificity of the drug effects and rules out nonspecific toxic effects on KC. BIOCHEM PHARMACOL 51;10:1315–1320, 1996.

KEY WORDS. antipsoriatic compound; drug effects; gene expression; growth regulators

The control of cell growth is a delicately balanced process, regulated by external signals (intercellular communication network) or the internal genetic program of an individual cell. The external signals are provided by growth factors, hormones, or cytokines that are synthesized by neighbouring cells or arrive *via* body fluids. The signals are usually transduced across the cell membrane by highly specific receptors that initiate a subsequent cascade of second messengers or protein kinase chains [1]. Ultimately, the message arrives in the cell nucleus where the expression of certain target genes is set for either proliferation or differentiation. In the hyperproliferative skin disease psoriasis, these processes are disturbed and some candidate genes are suspected of being involved in the pathogenesis of the disease when their expression is deregulated. Among these are the mitogenic cytokines TGF-alpha and interleukin-6 (IL-6) [2], the epidermal growth factor receptor EGF-R [3] and, most importantly, IL-8,‖ which is massively overproduced in psoriatic scales [4]. Inside the cell, some members of transduction cascades encoded by protooncogenes such as c-ras, c-raf, and HER-2, and growth-inhibiting gene products (e.g. the DNA-binding protein p53) could be causally involved in the development of the hyperproliferative phenotype of psoriasis. The disease can be effectively treated with drugs such as cyclosporin A [5] or tacrolimus FK506 [6], which are known to influence gene expression in T-cells when complexed to an intracellular binding protein (cyclophilin) and the protein phosphatase calcineurin [7]. Earlier, we demonstrated the presence of epidermal recep-

§ Corresponding author: G. Michel, Ph.D.; Hautklinik, Medizinische Einrichtungen der Heinrich-Heine-Universität Düsseldorf, Morrenstr. 5, Postf. 10 10 07, 40 001 Düsseldorf, Germany. FAX 49/211-811-7316.

‖ *Abbreviations:* IL-8, interleukin-8; 12(S)-HETE, 12(S)-hydroxytetraenoic acid; EtBr, ethidium bromide; KC, keratinocytes; PCR, polymerase chain reaction; GV, grey value.

Received 19 May 1995; accepted 18 December 1995.

tors for the signal mediators 12(S)-HETE and IL-8 [8, 9] and their disordered expression in psoriatic skin [10, 11]. Because psoriatic KC also produce large amounts of the ligand IL-8, we postulated an autocrine amplification loop involving IL-8/IL-8R in the growth stimulation of psoriatic epidermis. In addition, we showed a dose-dependent downregulation of the mRNA level, as well as the binding activity of type I IL-8R by FK506 [11]. Therefore, we investigated the effects of treatment with FK506 on the expression of growth-related genes in cultured normal human KC.

METHODS

Normal KC were isolated from newborn foreskin and cultured as described earlier [12]. Prior to the assays, the cells were kept overnight in medium containing only 0.5% fetal calf serum to minimize effects of serum-contained factors. Then, the cells were incubated in the presence of 5 μM FK506 for 3 hr. Controls were left untreated. Cytotoxicity of FK506 in the concentration range used here could be excluded in proliferation assays described in [11]. Skin biopsies were snap frozen in liquid nitrogen and homogenized to powder in a microdismembrator (Braun-Melsungen, Melsungen, Germany).

For semiquantitative mRNA-PCR, total RNA was isolated by acidic phenol extraction. After reverse transcription of 1 μg RNA from oligo-dT primers, specific amplification reactions were performed using a 1/20 aliquot of the RT reaction. Primers were synthesized according to the cDNA sequence published for the investigated genes (sequences given in 5′-3′ orientation; L, 5′ primer; R, 3′ primer):

HER-2 [13]: L: CAGTGCTTCAATTGCAGCCT
R: CAGGTGTCGATTTCCCACAA
c-ras [14]: L: GCTTCCTGTGTGTGTTTGCC
R: TCAGGAGAGCACACACTTGC
c-raf [15]: L: ACCCATTCAGTTTCCAGTCG
R: TTGATATCCTCAGTGTGGGC
p53 [16]: L: CGTCTGGGCTTCTTGCATTC
R: CTTGCGGAGATTCTCTTCCT
IL-8 [17]: L: AGTGCTAAAGAACTTAGATGTCA
R: TTCTCCACAACCCTCTG
IL-2 [18]: L: ATGTACAGGATGCAACTCCTGTCTT
R: GTCAGTGTTGAGATGATGCTTTGAC
β-actin [19]: L: AGAGATGGCCACGGCTGCTT
R: ATTTGCGGTGGACGATGGAG

For each primer pair, optimal amplification conditions were sought by dilution series (described in [11]). Optimal cycle numbers were determined for each product analogous to the example shown for the β-actin gene (Fig. 1). After 30–35 PCR amplification cycles (1 min 94°C, 1 min at T_m - 5°C), 1 min at 72°C) the products were visualized by EtBr-stained agarose gel electrophoresis. Band intensities from both treated vs untreated KC cultures, and also from lesional vs nonlesional skin, were monitored by densitometric scanning, standardized against β-actin signals from parallel reactions and plotted as "% of control" (Semiquantitative RT-PCR).

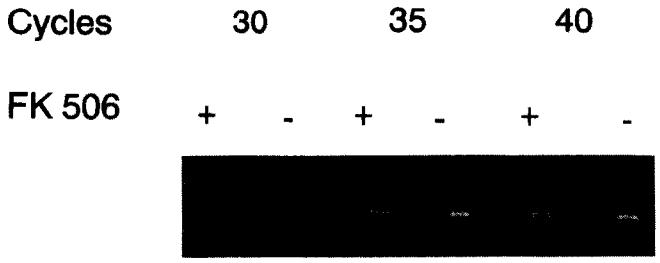

FIG. 1. Relation PCR-cycle number/signal strength. 30, 35, or 40 cycles of PCR reactions specific for β-actin were performed using reverse transcribed RNA from normal and FK506-treated KC.

For Northern blotting 10 μg of total RNA were denatured by heating to 50°C for 30 min in 10 mM sodium phosphate buffer (pH 6.6) containing glyoxal and DMSO. After electrophoresis, the RNA was transferred to a Nylon membrane. Hybridisation was carried out under stringent conditions at 42°C in 1 M sodium chloride, 10% dextran sulfate, 50% formamide, and 1% SDS. The p53 cDNA-probe was labelled by random priming (Megaprime Kit) in the presence of [32P]dCTP. Membranes were washed twice for 30 min in 0.1 × SSC, 1% SDS at 50°C. X-ray film (Kodak X-AR) was exposed for 3 days at −70°C. As control for equal RNA loading, the membrane was treated with Methylene blue (0.5 M sodium acetate, pH 5.2) to stain the ribosomal RNAs [20].

For immunocytochemistry, cells were grown on microscopic multichamber slides and treated with 5 μM FK506 for 3 hr. As positive control, the breast carcinoma cell line T-47D (ATCC Nr. HTB 133) was used, which constitutively expresses p53 (pers. commun. D. Niederacher, Düsseldorf, Germany). As negative control, T-47D cells were stained, omitting the p53-specific first antibody. The cells were fixed in methanol (3 min) and acetone (3 min) and washed in PBS. To inactivate cell-derived peroxidase activity, the slides were incubated in methanol/0.3% H_2O_2 for 30 min. Unspecific antibody binding was blocked in a mixture of 10 mL PBS/0.5 g milk powder/3 drops of normal goat serum from a commercial staining kit (Vectastain ABC kit, Camon, Wiesbaden, Germany). Then slides were incubated for 1 hr with mouse monoclonal antihuman p53 antibody (clone DO-1, diluted 1/100 in PBS/0.1% BSA). This antibody recognizes both wild-type and mutant p53. After washing, bound antibody was detected using the Vectastain ABC kit mentioned above, exactly as recommended by the manufacturer. It comprises a biotinylated secondary antibody, a peroxidase-coupled avidine-biotin complex, and a diaminobenzidine-substrate solution. Then, the samples were dehydrated and embedded in Eukitt. The staining intensity of the nuclei was quantitatively analyzed by a new microscopic "true colour image analysis system" (CIRES 7/14, Carl Zeiss, Jena, Germany). 10 optical fields (>350 nuclei) were evaluated for both FK506-treated and

untreated cells and 2 optical fields (>50 nuclei) for both positive and negative control. The integrated optical densities were plotted as mean GV.

Reagents

Reverse transcriptase was from Life Technologies (Eggenstein, Germany) and Taq polymerase from Pharmacia Biotech (Freiburg, Germany), both delivered with the recommended 10× reaction buffers. Specific primers were from MWG-Biotech (Ebersberg, Germany) and oligo-dT primers from Boehringer (Mannheim, Germany). Biodyne A transfer membrane was obtained from Pall (Dreieich, Germany). [32P]dCTP was from NEN-DuPont (Bad Homburg, Germany). The Megaprime-Kit was from Amersham-Buchler (Braunschweig, Germany). The cDNA-clone of p53 was from ATCC (Rockville, MD). FK506 was from Fujisawa (Munich, Germany). The mouse antihuman-p53 monoclonal antibody (AB-6, clone DO-1) was from Dianova (Hamburg, Germany). The Vectastain ABC kit was from Camon (Wiesbaden, Germany). All other chemicals were obtained from Sigma Chemie (Deisenhofen, Germany).

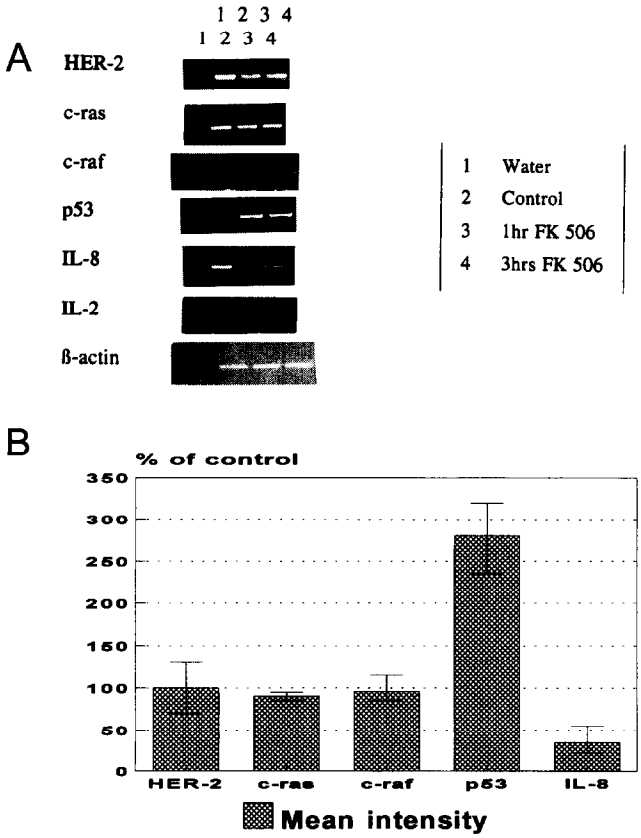

FIG. 2. Modulation of gene expression. (A) Band intensities of specific mRNA-PCR amplification products; and (B) semiquantitative densitometric evaluation thereof after treatment of cultured normal human KC with 5 µM FK506 for 3 hr. Bars represent mean of 4 independent experiments ± SD.

RESULTS

We present evidence for the modulation of gene expression in epidermal cells by the antipsoriatic drug FK506. For this purpose, we performed semiquantitative mRNA-PCR analyses. To ensure nonsaturated-phase PCR reactions, the appropriate cycle numbers were determined in preliminary experiments (Fig. 1). In addition, in the saturated phase the resulting signals should be equally strong in all samples. Therefore, in all experiments to be described, actual differences in signal strength by themselves exclude saturation of the reactions. As a negative control for false amplification signals, we performed PCR reactions specific for the IL-2 gene, which is not expressed in normal human KC.

Densitometric scanning of the photographed amplification products and normalization against β-actin signals from parallel reactions documented an 85% reduction in the IL-8 mRNA level after 1 hr and a 40–75% reduction after 3 hr of FK506 treatment (Fig. 2A and B). This effect was dose-dependent (Fig. 3A and B).

In contrast, in FK506-treated cells, the p53-specific RT-PCR signal was approximately 3-fold stronger than that from untreated controls. This tendency could be verified in a Northern blot of the same RNAs, hybridized against p53-cDNA (Fig. 4).

The expression of the protooncogenes Her-2, c-ras, and c-raf was not influenced by treatment with FK506.

Because p53 seems to be a target for FK506, we studied the expression of p53 in psoriatic skin. Biopsies from 3 patients revealed a reduction in p53 transcript levels by 10–88% in involved skin, compared to uninvolved or healthy skin (Fig. 5).

In full knowledge of the limitations of semiquantitative PCR with regard to the generation of absolute values for mRNA levels, we postulate that the observed differences reflect real changes that might be even more pronounced than the tendencies suggested by our data.

In a methodically different approach, we studied effects of FK506 on nuclear p53 expression using p53-specific monoclonal antibodies in immunocytochemistry (peroxidase-based permanent staining). The mamma carcinoma cell line T-47D used as a positive control showed dark staining of roughly the same intensity in all nuclei. When the p53-specific antibody was omitted, only very weak background staining could be observed (negative control). Comparison of treated KC with untreated controls clearly showed both an increased number of stained nuclei, as well as significantly darker staining, indicating increased p53 protein levels. For exact quantification of this induction, the slides were evaluated using the new "true colour image analysis system" CIRES (Carl Zeiss, Jena, Germany). Figure 6 shows the 4 distribution curves of GVs for positive control (black), negative control (white), untreated KC (light grey), and treated KC (dark grey). The GV distribution of FK506-treated cells (mean GV appr. 89) is clearly distinct from that of untreated cells (mean GV approximately 120).

TABLE 1. Integrated mean grey values calculated from image analysis of nuclei analysed for p53 expression

Class	Objects	Mean GV	SD
Untreated	352	120.46	27.24
FK506-treated	388	89.06	35.27
T-47D±	54	17.13	17.40
T-47D−	58	143.63	10.57

Table 1 summarizes the mean integrated optical densities and statistical analysis of these data.

DISCUSSION

The mechanism of action of FK506 in T-cells involves complex formation to intracellular binding proteins (FKBPs) and subsequent inhibition of a protein phosphatase (calcineurin). As a consequence, the transcription factor NF-AT is not dephosphorylated, cannot be translocated across the nuclear membrane, and gene expression (for example the IL-2 gene) cannot be initiated in T cells (7). Here, we present evidence that FK506 treatment leads to changes in the transcript levels of a number of genes in normal human epidermal cells as well.

In the present paper, we showed dose-dependent down-modulation of gene activity for the cytokine IL-8, which is overexpressed in psoriatic scales. In earlier work, we were able to demonstrate that the mRNA level for the IL-8 receptor (IL-8R) is also massively elevated in psoriatic skin

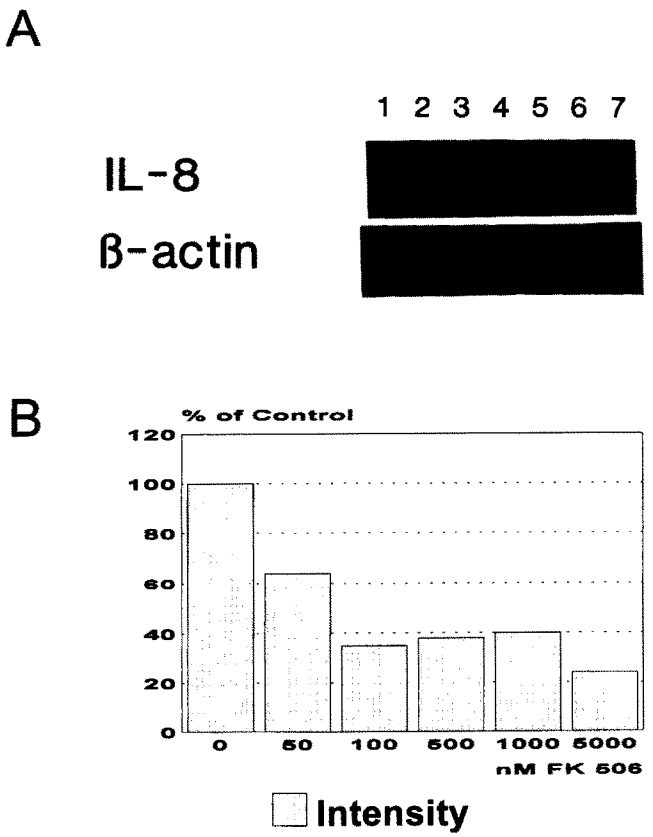

FIG. 3. Dose-response curve. (A) IL-8 gene expression after treatment with increasing concentrations of FK506 determined by specific mRNA-PCR. Concentrations used: 0 (Lane 1), 50 nM (2), 100 nM (3), 500 nM (4), 1000 nM (5), 5000 nM (6), water (7). (B) Bands were densitometrically scanned and plotted as % of control.

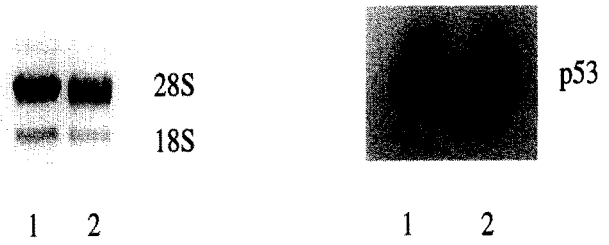

FIG. 4. p53 expression in FK506-treated KC cultures. Total RNA from untreated (lane 1) and FK506-treated (lane 2). KC was blotted on nylon membrane, stained with Methylene blue and, subsequently, hybridized under stringent conditions against p53-cDNA as described in Methods.

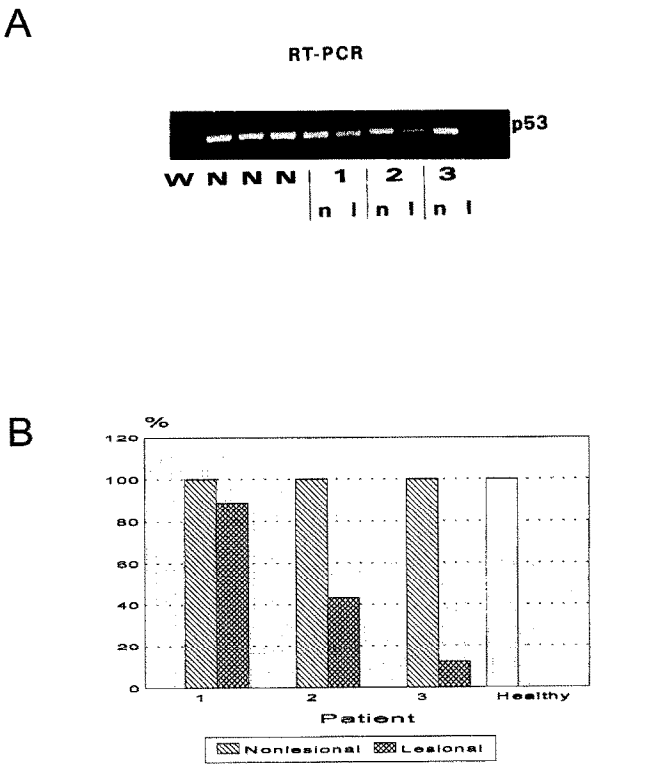

FIG. 5. p53 expression in psoriatic skin. (A) p53 gene expression was monitored by RT-PCR in skin biopsies from 3 normal healthy volunteers (N) and 3 psoriatic patients (n, nonlesional; 1, lesional). (B) Bands were densitometrically scanned and plotted as % of normal.

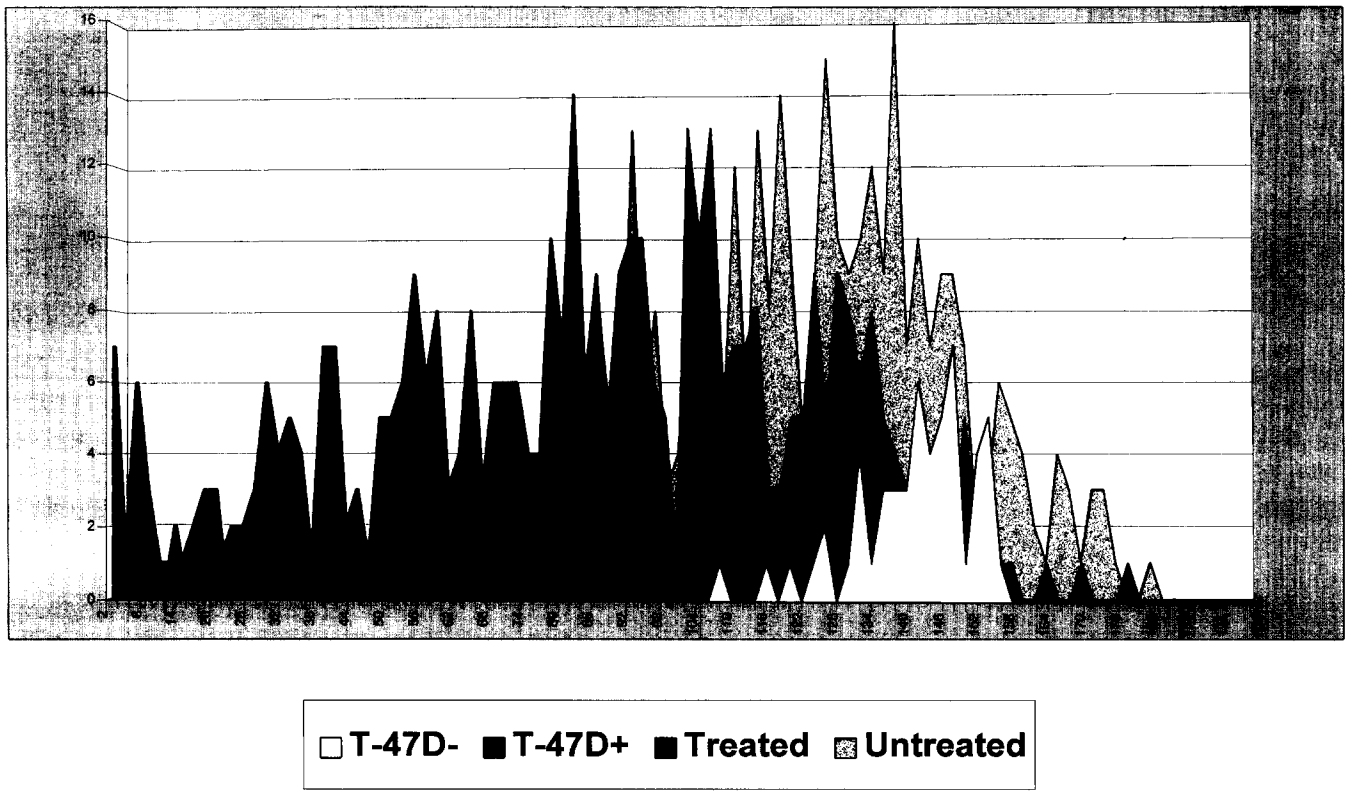

FIG. 6. True colour image analysis of p53-immunocytochemistry. Epidermal cells were untreated (light grey curve) or treated with 5 μM FK506 (dark grey curve) and probed for p53 expression with specific mABs as described in Methods. Positive control (black curve), T-47D mamma-carcinoma cells (T-47D±). Negative control (white curve): T-47D cells, where p53-specific primary Ab was omitted during staining (T-47D−). Staining intensity of the nuclei was determined using the new CIRES system (Carl Zeiss, Jena, Germany). y-axis, number of nuclei; x-axis, Mean GV = Mean grey value (0 = Black, 200 = white).

and that IL8R expression is inhibited by FK506 in normal KC [11]. (Interestingly, other antipsoriatic drugs, such as the vitamin D3 derivative calcipotriol, show similar effects [21]). The relevance of these data was recently confirmed by a clinical report from Lemster *et al.* [22]. The authors describe downregulation of IL-8 mRNA in involved psoriatic skin after systemic treatment of patients with FK506. IL-8 receptor transcript levels in the same patients returned to levels comparable to normal, healthy skin, which is in excellent correlation with our *in vitro* data described in [11]. In conclusion, we deduce a model where, in an autocrine loop, the pathological overexpression of the IL-8/IL-8R system causes hyperproliferation of psoriatic keratinocytes. In addition to these data, we present evidence for decreased transcript levels of the cell cycle inhibitor p53 in psoriatic plaques and elevated expression after FK506 treatment in cultured epidermal cells. The increase was clearly evident on the mRNA as well as protein levels. These data are in excellent accordance with a report by Healy *et al.* [23], who find increased p53 mRNA and protein *in vivo* in normal human skin after application of the potent antipsoriatic drug dithranol. The observation of transcriptional induction of p53 is interesting insofar as changes in the amount of p53-protein have been mainly ascribed to posttranslational stabilization of mutated p53, rather than to transcriptional induction. Because the antibody used in our immunocytochemical experiments does not discriminate between wild-type and mutated forms of the p53 protein, posttranslational modification could be responsible for the enhanced protein levels demonstrated here. However, this type of regulation of gene expression does not require changes in transcription, as shown above by the RT-PCR and Northern blot experiments. Therefore, we conclude that changes in the transcription rate of the p53 gene are involved in the pathogenesis of psoriasis.

In conclusion, we show modulation of the expression of three different growth-related genes (IL-8, IL-8R type I, and p53) by a drug *in vitro* that counters the corresponding dysregulation in psoriatic skin *in vivo*. This could well be an explanation for both the mechanism of action of FK506 and for the pathomechanism of psoriasis: because IL-8 is a mitogen for KC and p53, in contrast, is known to inhibit cell cycle progression, their opposite regulation by FK506 reflects the same tendency to slow down cell growth.

In contrast, the mitogenic signal transduction pathways involving the protooncogene products HER-2, c-ras, or c-raf were unaffected by FK506 treatment.

The differential modulation of unrelated genes underlines the specificity of the observed effects and rules out any toxic effects of the substance.

References

1. Rosenbach T and Czarnetzki B, Signal transduction pathways in keratinocytes. *Exp Dermatol* **1:** 59–66, 1992.
2. Menter A and Barker JN, Psoriasis in practice. *Lancet* **338:** 231–234, 1991.
3. Rothe M and Falanga V, Growth Factors. *Arch Dermatol* **125:** 1390–1398, 1989.
4. Christophers E, Schröder JM and Mroweitz U, Identification of two endogenous neutrophil-activating peptides in psoriatic skin and inflammatory cells: C5ades arg and NAP. *Dermatologica* **179 Suppl 1:** 9–15, 1989.
5. Meingassner JG and Stütz A, Immunosuppressive macrolides of the type FK506: a novel class of topical agents for treatment of skin diseases? *J Invest Dermatol* **6:** 851–855, 1992.
6. Jegasothy BV, Ackerman CD, Todo S, Fung JJ, Abu Elmagd K and Starzl TE, Tacrolimus (FK506)—a new therapeutic agent for severe recalcitrant psoriasis. *Arch Dermatol* **128:** 781–785, 1992.
7. Schreiber SL and Crabtree GR, The mechanism of action of cyclosporin A and FK506. *Immunol Today* **13:** 136–142, 1992.
8. Arenberger P, Kemény L and Ruzicka T, Characterization of high-affinity 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) binding sites on normal human keratinocytes. *Epith Cell Biol* **2:** 1–6, 1993.
9. Michel G, Kemény L, Peter RU, Beetz A, Ried C, Arenberger P and Ruzicka T, Interleukin-8 receptor-mediated chemotaxis of normal human epidermal cells. *FEBS Lett* **305:** 241–243, 1992.
10. Arenberger P, Kemény L, and Ruzicka T, Defect of epidermal 12(S)-hydroxyeicosatetraenoic acid receptors in psoriasis. *Eur J Clin Invest* **22:** 235–243, 1992.
11. Schulz BS, Michel G, Wagner S, Süss R, Beetz A, Peter RU, Kemény L and Ruzicka T, Increased expression of epidermal IL-8 receptor in psoriasis. Down-regulation by FK-506 in vitro. *J Immunol* **151:** 4399–4406, 1993.
12. Süss R, Arenberger P, Gross EC and Ruzicka T, Regulation of 12(S)-hydroxyeicosa-tetraenoic acid (12(S)-HETE) binding sites on human epidermal cells by interferon-gamma. *Exp Cell Res* **191:** 204–208, 1990.
13. Coussens L, Yang Feng TL, Liao YC, Chen E, Gray A, McGrath J, Seeburg PH, Libermann TA, Schlessinger J, Francke U, *et al.*, Tyrosine kinase receptor with extensive homology to EGF receptor share chromosomal location with neu oncogene. *Science* **230:** 1132–1139, 1985.
14. Capon DJ, Chen EY, Levinson AD, Seeburg PH and Goeddel DV, Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. *Nature* **302:** 33–37, 1983.
15. Bonner TI, Oppermann H, Seeburg P, Kerby SB, Gunnell MA, Young AC and Rapp UR, The complete coding sequence of the human raf oncogene and the corresponding structure of the c-raf-1 gene. *Nucleic Acids Res* **14:** 1009–1015, 1986.
16. Zakut-Houri R, Bienz Tadmor B, Givol D and Oren M, Human p53 cellular tumor antigen: cDNA sequence and expression in COS cells. *EMBO J* **4:** 1251–1255, 1985.
17. Matsushima K and Oppenheim JJ, Interleukin 8 and MCAF: Novel inflammatory cytokines inducible by IL-1 and TNF. *Cytokine* **1:** 2–12, 1989.
18. Taniguchi T, Matsui H, Fujita T, Takaoka C, Kashima N, Yoshimoto R and Hamuro J, Structure and expression of a cloned cDNA for human interleukin-2. *Nature* **302:** 305–310, 1983.
19. Nakajima-Iijima S, Hamada H, Reddy P and Kakunaga T, Molecular structure of the human cytoplasmic beta-actin gene: interspecies homology of sequences in the introns. *Proc Natl Acad Sci USA* **82:** 6133–6137, 1985.
20. Wilkinson M, Doskow J and Lindsey S, RNA blots: staining procedures and optimization of conditions. *Nucleic Acids Res* **19:** 679, 1990.
21. Kemény L, Kenderessy AS, Olasz E, Michel G, Ruzicka T and Dobozy A, The interleukin-8 receptor: a potential target for antipsoriatic therapy. *Eur J Pharmacol* **258:** 269–272, 1994.
22. Lemster BH, Carroll PB, Rilo HR, Nikaein A and Thomson AW, IL8/IL-8 receptor expression in psoriasis and the response to systemic tacrolimus (FK506) therapy. *Clin Exp Immunol* **99:** 148–154, 1995.
23. Healy E, Reynolds NJ, Smith MD, Campbell C, Far PM and Rees JL, Dissociation of erythema and p53 protein expression in human skin following UVB irradiation, and induction of p53 protein and mRNA following application of skin irritants. *J Invest Dermatol* **103:** 493–499, 1994.