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Original Article

The changes in expression of Ki-67, and CD31 in psoriatic lesions before and after etanercept treatment

牛皮癬皮損在生物製劑依那西普治療前後的Ki-67及CD31表現水平變化

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Background: Although the effectiveness of etanercept in the treatment of psoriasis is very well-established, the mechanism of action is poorly understood. It was suggested that the therapeutic effect of etanercept in psoriasis could be mediated by the inhibition of TNF expression. Although clinical trials have sometimes demonstrated a dramatic response to TNFα inhibition, only a few studies have examined the changes occuring in the skin upon TNFα inhibition. ***Objectives:*** The aim of our study was to investigate the different effects of etanercept on cell proliferation, inflammatory infiltrate, and angiogenesis in psoriasis, and to clarify the mechanism by which etanercept exerts its therapeutic effects. ***Methods:*** Clinical response, the morpho-phenotypic changes, epidermal thickness, mitotic count were analysed and the expression of CD31, proliferative marker as Ki-67, were evaluated by immunohistochemical techniques in lesional psoriatic epidermis, before and after treatment with etanercept in 11 patients. ***Results:*** In post-treatment biopsies, a decrease in the degree of epidermal hyperplasia and a significant reduction in the severity of the inflammmatory infiltrate ($p<0.05$) were observed. In addition, CD31 expression was significantly decreased in the dermal cellular infiltrate, ($p<0.05$). Ki67 expression was significantly suppressed concurrently in about 90% of cases ($p<0.01$). ***Conclusions:*** We suggest that etanercept may have an inhibitory effect on an initial integral component of the pathways that leads to psoriasis. Immunopharmacologic intervention in inflammatory event has the potential to improve psoriasis. Inhibition of neovascularisation may be another mechanism of action of etanercept.

背景：雖然依那西普治療牛皮癬的功效衆所周知，但當中的作用機制卻所知甚少。有專家認爲依那西普治療牛皮癬的療效，可能來自通過對腫瘤壞死因子表達抑制的調節。雖然臨床試驗不時展示α腫瘤壞死因子抑制的顯著療效，但只有少數研究曾探究當中α腫瘤壞死因子抑制在皮膚上所造成的變化。**目標**：本研究的目的是調查依那西普在牛皮癬治療中對細胞增殖、炎性滲入和血管

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生成的不同效果，並闡明依那西普的治療效果是取決於那一種機制。**方法**：本研究對十一名接受依那西普治療的牛皮癬患者，進行治療前後的表皮皮損分析，當中包括臨床反應、形態表現的變化、表皮厚度和核分裂數，並使用免疫組織化學 技術探測皮損當中的 CD31 及細胞增殖標記Ki-67的表現水平。**結果**：在治療後的活檢樣本中，可見表皮增生減少，而炎性滲入嚴重程度更是大幅減少（$p<0.05$）。此外，皮膚細胞滲入中的CD31表現水平明顯下降（$p<0.05$）。同時，約九成患者的Ki67表現水平，受到顯著抑制（$p<0.01$）。**結論**：我們認爲依那西普可能對牛皮癬初始形成過程中的元件有著抑制作用。在牛皮癬炎症過程中，免疫藥理的干預有著潛在的治療價值。此外，抑制新生血管形成亦可能是依那西普的另一作用機制。

Keywords: CD31, Etanercept, Ki-67, psoriasis

關鍵詞：CD31 ，依那西普， Ki-67 ，牛皮癬

Introduction

Psoriasis is a chronic inflammatory T-cell mediated immune dermatosis, characterised by a high epidermal cell turnover, which results in a typical epidermal hyperplasia.[1-3] The pathogenesis of psoriasis is linked to the recruitment and activation of several types of leucocytes, and to a T-cell-dependent inflammatory process in skin that accelerates the growth of epidermal and vascular cells in psoriatic lesions. The most important processes in immunological activation are Langerhans cell and T cell activation, differentiation and expression of type 1 T helper cells, selective trafficking of activated T cells to skin, and induction of an inflammatory cytokine and chemokine cascade in the skin lesions.[4,5] The platelet endothelial cell adhesion molecule-1 (PECAM-1)/CD31 is a newly assigned member of the immunoglobulin superfamily of cell adhesion molecules.[6,7] It is highly expressed at the lateral junctions of endothelial cells and at a lower density on the surfaces of neutrophils, monocytes, platelets, natural killer cells, T and B cell subsets, and mast cells. CD31 has been implicated to be involved in neutrophil recruitment in vivo, neutrophil and monocyte chemotaxis, and transendothelial migration of monocytes and neutrophils in vitro.[8] In addition, it also plays a role in angiogenesis, platelet aggregation and homeostasis, and the maintenance of the vascular endothelial barrier function. CD31 is highly expressed in all endothelial cells of existing and newly-formed blood and lymphatic vessels, making it a useful and commonly-used endothelial cell marker.[9,10]

In psoriasis, assessment of vasculature in the papillary dermis with monoclonal antibody JC/70A specific for the endothelial marker CD31 demonstrated a fourfold increase in endothelial surface area of lesional skin compared with non-lesional skin.[9] The proliferative markers such as proliferating cell nuclear antigen and nuclear proliferation marker Ki-67, which are nuclear proteins associated with cell cycle, are increased in psoriatic when compared with normal skin.[11,12]

Etanercept is a recombinant tumour necrosis factor (TNF)-receptor fusion protein, which consists of two extracellular ligand-binding domains of the human p75 tumour necrosis factor receptor fused to the Fc portion of human IgG1. Although clinical trials have sometimes demonstrated a dramatic response to TNFα inhibition, only a few studies have examined the changes occuring in the skin upon TNFα inhibition. Preliminary studies showed a decrease in cellular infiltration with normalisation of keratinocyte differentiation in the skin.[13] More recent studies have highlighted the vascular changes that occur in the skin upon TNFα inhibition:[14] there is a reduction in vessel number, expression of vascular endothelial growth factor (VEGF), and expression of both angiopoietin-1 and angiopoietin-2. These studies show that the reduction in cellular infiltration may be secondary

to a reduction in both angiogenesis and in cellular trafficking.

The aim of our study was to investigate the different effects of etanercept treatment on cell proliferation, inflammatory infiltrate, and angiogenesis in psoriasis, and to clarify the mechanism by which etanercept is effective against psoriasis. For this purpose, we evaluated the clinical response, the morpho-phenotypic changes, epidermal thickness, mitotic count, the expression of CD31, the proliferative marker such as Ki-67 in lesional psoriatic epidermis, before and after treatment with etanercept in 11 patients with psoriasis.

Material and methods

Patients

Eleven patients (8 males, 3 females) with clinically active psoriasis treated with subcutaneous etanercept 25 mg twice weekly (Enbrel®, Wyeth Pharmaceuticals, Havant, England) were included in the study. Active psoriasis was defined as plaques increasing in size and number at the time of investigation. The ages of patients ranged from 22 to 69 years (mean 47.50±13.52). The duration of psoriasis ranged from two to 25 years (mean 9.80±6.52 years). All topical and systemic treatments had to be discontinued two weeks to two month respectively, prior to the study. The severity of psoriasis was assessed using the psoriasis area and severity index (PASI), a well established grading system that takes into account erythema, induration and scaling of psoriatic plaques and skin surface area affected by psoriasis.[15] In the patients studied, the PASI scores ranged from 7.1 to 23.5 (mean 12.51±5.39). All patients gave their informed consent to donate skin specimens for investigation.

Biopsies

Two 4-mm punch biopsies were obtained under local xylocaine anesthesia from the lesional skin of each patient at the same affected site before, and at 1.5 months of etanercept therapy. Formalin-fixed skin fragments were embedded in paraffin and processed routinely. Hematoxyline-eosin sections were used to grade the severity of psoriatic lesions. The severity of the inflammatory infiltrate was measured as (1) mild (very few inflammatory cells), (2) discrete (perivascular inflammatory infiltrate with occasional exocytosis), (3) moderate (perivascular inflammatory infiltrate with marked exocytosis), and (4) severe (perivascular and diffuse dermal infiltrate with severe exocytosis and intraepidermal pustule formation). Cell turnover was evaluated by calculating the number of the mitotic figures in ten fields (at a magnification of x400). The epidermal basal layer was also included in the counts.

Morphometric evaluation

Epidermal thickness was measured in sections stained with hematoxyline and eosin. Nikon Digital sight DS-L1 attached to Nikon Eclipse E 600 microscope was used. The mean distance from the bottom of the stratum corneum to the bottom of the rete ridge was calculated from the measurement of five fields (at a magnification of x100).

Immunohistochemistry

Five-µm-thick sections were prepared from the parafin tissues and evaluated to quantify Ki-67 and CD31 protein expression. The avidin-biotin complex immunoperoxidase staining system was used.

Primary antibodies used were: Ki-67 Ab-2 (Diagnostic Biosystems, Clone SP6) rabbit monoclonal antibody with a dilution of 1:25; CD31/PECAM-1 (endothelial cell marker) Ab-1 (Diagnostic Biosystems Clone JC/70A) mouse monoclonal antibody with a dilution of 1:30. The sections were de-paraffinised in xylene through ethanol to phosphate-buffered saline (PBS; pH 7.2). To block endogenous peroxidase activity, 3% hydrogen peroxide was applied for 30 minutes. The slides incubated in citrate buffer were heated in a microwave oven

for 5 minutes. After waiting for 20 minutes, they were removed, and ultra V block was added. Primary antibodies were applied and incubated for one and a half hours in a moist chamber at 4°C. The slides were subsequently incubated in biotinylated goat antipolyvalent for 10 minutes and in streptavidin peroxidase for 20 minutes. Finally, 3-amino-9-ethylcarbazole (AEC) substrate system (ThermoScientific Labvision Corporation, Fremont, CA, USA) was applied for about 3 minutes. After incubation, the sections were rinsed with distilled water and tap water. The tissue was counterstained with Mayer's hematoxyline. All slides were covered with a cover-slip after mounting in buffered glycerin

Immunohistochemical determination

Ki-67

The level of staining was scored on the following 4-point scale: no staining (grade 0), moderate focal/faint diffuse staining (grade 1), strong focal/ moderate diffuse staining (grade 2), and strong diffuse staining (grade 3).

CD31

A semi-quantitative scale was used to evaluate the staining: (0) no staining; (1) weak staining, limited to papillary endothelium; (2) moderate, diffuse endothelial staining; (3) severe, diffuse endothelial staining.

Statistical analysis

Data from image analysis studies were expressed as mean±standard error or mean rank, as appropriate. We also calculated 95% confidence intervals for the mean difference. Correlation was determined by Spearman's rank correlation, depending on the normality of the data for some variables. Continuous variables were tested for normal distribution using the Kolmogorov-Smirnov normality test, comparisons between groups (psoriatic patients before and after therapy, and non-psoriatic patients) were assessed using the Wilcoxon signed-rank test and paired t test. P-values of less than 0.05 were considered statistically significant. The statistical analysis was performed by using SPSS statistical software (Microsoft, Chicago, Ill., USA).

Results

Clinical and histological examination

An overall favourable clinical response to etanercept was seen in our patients. Despite significant improvement in all patients, complete clearing was not achieved in any case after 1.5 months of therapy. The patients tolerated the therapy well. There were no significant adverse effects. The histological examination of lesional skin revealed a decrease in inflammmatory cells and progressive loss of elongated rete pegs with the return of a normal stratum corneum.

Immunohistochemistry

CD31 protein was detected in vascular endothelial cells (Figure 1a) and Ki-67 protein was observed in the epidermal cells (Figure 2a). The mean±standard deviation of epidermal thickness was significantly greater in psoriatic skin before therapy (4009.87±1270.08 μm) than after therapy (1739.19±705.66 μm) ($p<0.001$) (Table 1). Although the turn-over median value of epidermis before therapy (a mitotic count of 3.00) decreased to 0.00 after therapy, the difference was not statistically significant ($p>0.05$).

Examination of biopsies obtained after therapy showed that there were reductions in the inflammatory infiltrate, the expression of CD31, and number of Ki-67 positive cells in comparison to pretreatment lesions (Figures 1a and 1b and Figures 2a and 2b, Table 2). A remarkable reduction in the number of Ki-67 stained nuclei was demonstrated ($p<0.01$). In the vascular endothelium of involved psoriatic skin, a significantly decreased expression of CD31 was noted after treatment ($p<0.05$).

Discussion

Psoriasis is a chronic inflammatory skin disorder affecting 2-4% of the world's population. The disease is characterised by epidermal hyperproliferation and inflammation. Psoriasis is considered to be a genetically programmed disease of dysregulated inflammation, which is driven and maintained by multiple components of the immune system. The pathologic collaboration between innate immunity and acquired immunity results in the production of cytokines, chemokines, and growth factors that contribute to the inflammatory infiltrate seen in psoriatic plaques. The aggravated state of innate immunity is represented by the activity of natural killer T cells, dendritic cells, neutrophils and keratinocytes, leading to the recruitment and

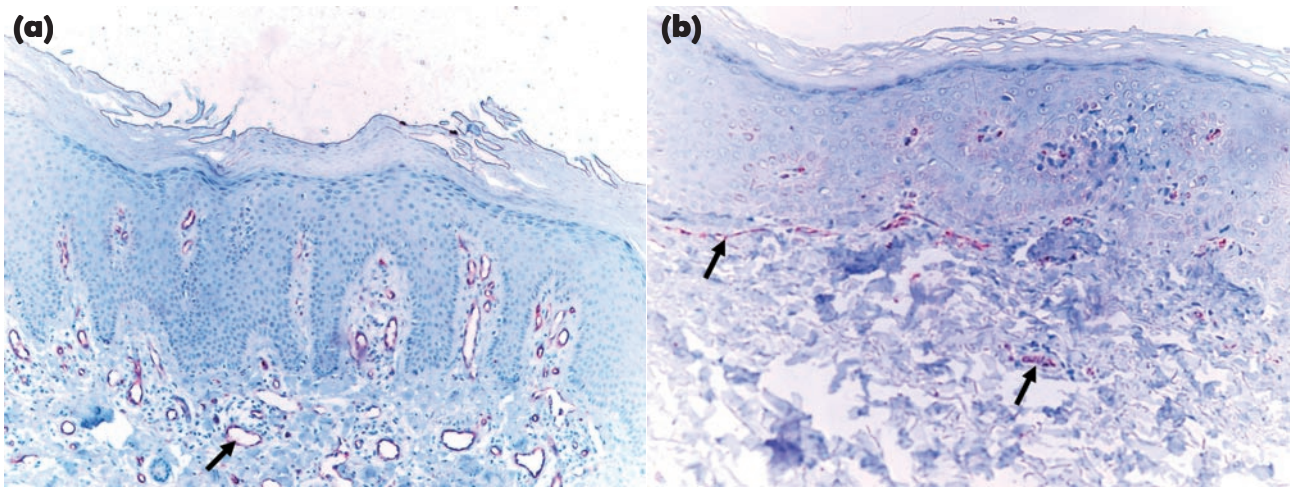

Figure 1. (a) Immunostaining of microvasculature in psoriatic lesional skin with CD31 before treatment with etanercept (CD31, x400). (b) There is a marked reduction of CD31+ cells following etanercept treatment in dermal vasculature (CD31, x400).

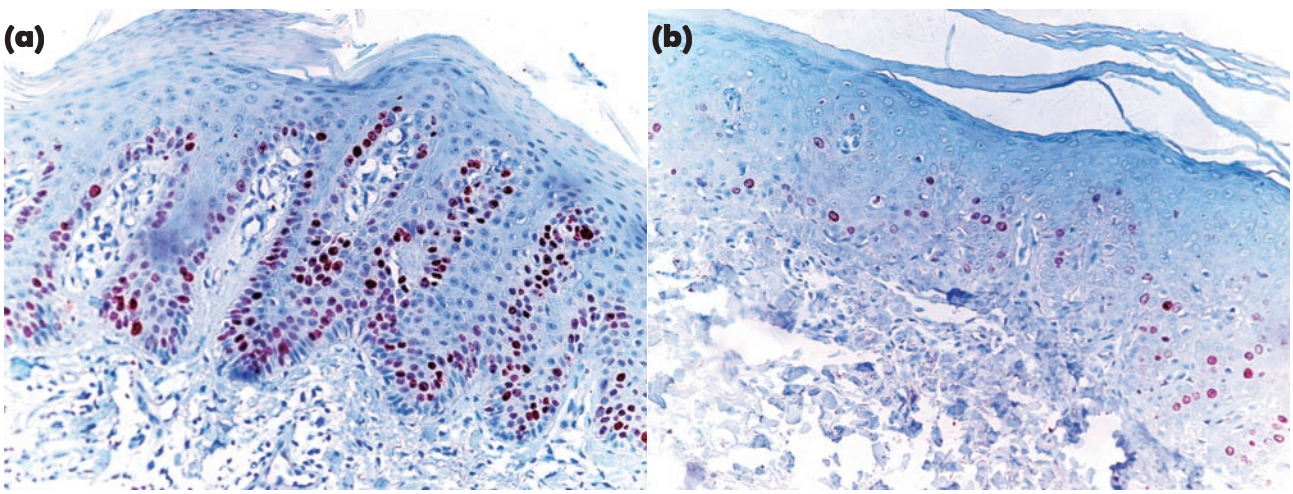

Figure 2. (a) Ki-67-positive cells were observed in the epidermal cells in psoriatic lesional skin before treatment with etanercept (Ki-67, x400). (b) Immunopositivity for Ki-67 is markedly reduced with etanercept therapy (Ki-67, x400).

Table 1. Mean ± standard error of epidermal thickness in psoriatic patients before and after therapy

	Before therapy Mean ± standart deviation	After therapy Mean ± standart deviation	Paired sample t test significance (p values)
Epidermal thickness	4009.87±1270.08 μm	1739.19±705.66 μm	<0.001

Table 2. The statistical results of changes in inflammatory infiltrate, turn-over and the expression of Ki-67, and CD31 in psoriatic patients before and after etanercept therapy. Results are shown as mean rank. P-values are also presented

	Median (Minimum-Maximum)	Wilcoxon t test significance (P values)
Before Therapy Ki-67	3.00 (2.00-3.00)	<0.01
After Therapy Ki-67	1.50 (1.00-2.00)	
Before Therapy CD-31	3.00 (1.00-3.00)	<0.05
After Therapy CD-31	1.00 (1.00-2.00)	
Before Therapy Turn-over	3.00 (1.00-10.00)	>0.05
After Therapy Turn-over	0.00 (0.00-6.00)	
Before Therapy Inflammation	2.00 (2.00-3.00)	<0.01
After Therapy Inflammation	1.00 (0.00-1.00)	

activation of preferentially type 1 T cells, possibly in an antigen-independent way.[3-5]

Tumour necrosis factor-alpha is a cytokine produced by lymphocytes and macrophages, two types of white blood cells. It mediates the immune response by increasing the transport of white blood cells to sites of inflammation, and through additional molecular mechanisms which initiate and amplify inflammation. Etanercept is a drug that treats autoimmune diseases by the inhibition of TNF. Etanercept is a fusion protein produced through expression of recombinant DNA. It is a product of a DNA "construct" engineered to link the human gene for soluble TNF receptor 2 to the gene for the Fc component of human immunoglobulin G1 (IgG1). Expression of the construct produces a continuous protein "fusing" TNF receptor 2 to IgG1. Production of etanercept is accomplished by the large-scale culturing of cells that have been "cloned" to express this recombinant DNA construct. It is a large molecule, with a molecular weight of 150 kDa., that binds to TNFα and decreases its role in disorders involving excessive inflammation in humans and other animals, including psoriasis and psoriatic arthritis, and, potentially in a variety of other disorders mediated by excessive TNFα. This therapeutic potential is based on the fact that TNF-alpha is the "master regulator" of the inflammatory response in many organ systems.[16,17] It reduces the effect of naturally present TNF, and hence is a TNF inhibitor, functioning as a decoy receptor that binds to TNF. There are two types of TNF receptors: those found embedded in white blood cells that respond to TNF by releasing other cytokines, and *soluble* TNF receptors which are used to deactivate TNF and blunt the immune response. In addition, TNF receptors are found on the surface of virtually all nucleated cells. Etanercept mimics the inhibitory effects of naturally occurring soluble TNF receptors, the difference

being that etanercept, because it is a fusion protein rather than a simple TNF receptor, has a greatly extended half-life in the bloodstream, and therefore a more profound and long-lasting biologic effect than the naturally occurring soluble TNF receptor.[18,19]

Although clinical trials have sometimes demonstrated a dramatic response to TNFα inhibition, only a few studies have examined the changes occuring in the skin upon TNFα inhibition.[14] Expansion of the dermal microvasculature is a prominent feature of psoriasis. Although the pathogenetic process resulting in vascular morphological changes remains unclear, considerable evidence suggests the involvement of angiogenesis.[20] In our study, CD31 expression on dermal cellular infiltrate was decreased after etanercept treatment. Psoriatic microvasculature changes are related to improvement of psoriasis plaques.[2] But there have been few studies on the antiangiogenic effects of antipsoriatic drugs.[14,21] We already showed that the inhibition of neovascularisation could be another mechanism of action of methotrexate in psoriatics.[2] Noborio et al observed that CD31 expressions decreased significantly after pulsed dye laser therapy in psoriatic lesions.[22] It was shown that based on the histological change of synovium, treatment by etanercept could be involved in vascular and cell proliferations with inhibition of the expression of CD68 and MMP-3 in the synovium of rheumatoid arthritis patients, suggesting that the inhibition of neovascularisation may be another action of etanercept in psoriasis treatment.[23]

Some proliferative markers are increased in psoriasis, when compared with normal skin. Various proliferative indices, such as the mitotic count, and markers, like Ki-67, have been evaluated in various fields of pathology to assess the rate of cell proliferation.[2,11,12] Miracco et al found that Ki-67 expressions decreased after ciclosporin treatment in psoriatics.[11] We have already indicated that Ki-67 expression was suppressed after methotrexate treatment in patients with psoriasis.[2] Recently, Gambichler et al evaluated whether etanercept plus narrowband ultraviolet B phototherapy was superior to etanercept monotherapy at 6-week therapy of psoriasis. They found that Ki-67 expressions did not differ significantly between the two treatments.[24] In 2010, Van der Velden et al observed that the number of Ki-67+ cells was decreased after topical calcipotriol/betamethasone dipropionate treatment in psoriatics.[25] In 2009, Kvist et al indicated that topical calcipotriol/betamethasone dipropionate and calcipotriol treatments reduced epidermal thickness, Ki-67 and cytokeratin 16 expression in the murine psoriasis xenograft model.[26] Adişen et al showed that calcipotriol and methylprednisolone aceponate decreased the p53 and Ki-67 expressions in psoriatics.[27] Van Duijnhoven et al also observed that treatment of psoriasis with short-term topical steroids resulted in a trend towards normalisation of Ki-67 antigen.[28] Ormerod et al found a significant reduction in proliferating cells (stained by Ki-67) in the epidermis with topical sirolimus treatment compared with controls.[29] Smit et al concluded that systemic treatment of psoriatic patients with bexarotene suppressed Ki-67 expression.[30] Vincek et al observed that in the skin biopsies of the psoriatic skin, the fraction of Ki-67-positive cells did not decrease after infliximab infusion and was comparable to the psoriatic skin before the treatment, although the epidermal thickness was decreased.[31] Jang et al found that retinoic acid treatment reduced Ki-67 expressions correlated with telomerase activity.[32]

Recently, Avramidis et al showed that etanercept caused a statistically significant time-dependent reduction in the number of dermal blood vessels, the number of CD31+ cells and VEGF in psoriatic lesions, with induction of endothelial cell apoptosis and statistically significant upregulation of antiangiogenic factor thrombospondin-1 in psoriatic vessels. They also showed significant reduction of NF-κB, Bcl-2 and Bcl-xL expression in endothelial cells during treatment. These changes were accompanied by a marked clinical response.[33] The pattern of reduced VEGF in relation to increased angiopoietin-2 in synovial

tissue suggests that vascular regression is a potential mechanism underlying the anti-angiogenic effect of infliximab in psoriatic arthritis.[34] In-vivo reduction of cutaneous en-plaque capillaries using intra-vital videocapillaroscopy analysis and VEGF expression by the etanercept is associated with improvement of psoriasis.[35] Several studies have shown that TNF-α can directly induce anti-apoptotic proteins in endothelial cells, and keratinocytes.[36] Inhibition of the nuclear factor-κB signalling pathway (NF-κB) is one of the major mechanisms by which anti- TNF-α therapy induces cell apoptosis.[37] Treatment with etanercept selectively induces apoptosis of dermal myeloid dendritic cells in psoriatic plaques before clinical and histological clearance. It has been hypothesised that etanercept inhibits activation / phosphorylation of NF-κB and induces dermal dendritic cell apoptosis via down-regulation of anti-apoptotic proteins Bcl-xL and Bcl-2.[38]

Ki-67, CD-31 and TNF-alpha expressions were significantly higher in psoriatic epidermis than in normal epidermis. In our study, a decrease of the degree of epidermal hyperplasia and a significant reduction in the severity of the inflammmatory infiltrate was observed in post-treatment biopsies. The effect of etanercept on Ki-67 expression was more pronounced than on CD31 expression. Ki67 and CD31 expression decreased concurrently in about 90% of cases. This study reported a rapid onset of clinical and histological improvement in psoriasis after etanercept treatment. These histological effects were seen within six weeks and were correlated with clinical improvement. Our results showed that the expression of CD31, and Ki-67 in psoriatic lesions was inhibited by etanercept therapy. Our findings were in line with these observations, suggesting that a concerted regulation of several angiogenic and inflammatory regulators by etanercept plays a significant role in the involution of psoriatic skin. We have shown that etanercept exerts a rapid and measurable anti-inflammatory effect on the psoriatic skin of patients with psoriasis. The clinical efficacy of etanercept therapy is well-established in psoriasis, but the exact pathophysiological mechanisms of this action in psoriatic skin are not entirely clear. In the present study, we provide additional information on the effect of etanercept on blood vessels in psoriasis and the mechanisms related to proliferative regression during treatment. Further work is necessary to determine the mechanisms by which etanercept inhibits angiogenesis, which is now considered to be an angiogenesis-dependent disease. Novel and selective therapeutic strategies in the treatment of psoriasis may be developed on this basis.

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