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ORIGINAL ARTICLE

Sequential Histological and Immunohistochemical Assessment of Proliferation and Apoptotic Markers During Treatment of Psoriasis With Antitumor Necrosis Factor α (Infliximab)

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Abstract. *Background and objectives.* Psoriasis is an inflammatory skin disease of immunologic nature that is mediated by T-helper-1 cytokines. Clinical response to treatment with antitumor necrosis factor (TNF) a antibodies (infliximab) has been significant; however, the mechanisms for clearance of lesions have not been elucidated. The aim of the present study was to assess variations in the histology and expression of proliferation and apoptotic markers in sequential skin biopsies of patients with psoriasis treated with infliximab.

Material and methods. We studied skin biopsies (of lesioned and healthy skin) from 3 patients with extensive moderate-to-severe psoriasis (mean psoriasis area and severity index [PASI] score, 35) treated with intravenous infliximab infusions (5 mg/kg) at weeks 0, 2, and 6. Biopsies were taken on days 0, 14, and 28, and were processed for conventional histological and immunohistochemical study. The apoptotic markers used were TP53, B-cell lymphoma 2 protein, anticaspase 3, and anticaspase 8. The cell proliferation marker used was Ki67.

Results. Treatment with infliximab was associated with a significant clinical improvement in 3 patients (mean PASI score, 21.6 at 14 days and 13.9 at 6 weeks), which correlated with the progressive disappearance of histological lesions with a decrease in epidermal proliferation. However, apoptosis was not observed, and the samples tested negative for anticaspase antibodies. Expression of TP53 decreased 2 weeks after starting treatment, and was similar to that in normal skin at 28 days.

Conclusions. Clinical and histological response of psoriasis to infliximab was not associated with a significant increase in the apoptotic markers assessed.

Key words: psoriasis, tumor necrosis factor a, histology, immunohistochemistry, apoptosis.

EVALUACIÓN HISTOLÓGICA E INMUNOHISTOQUÍMICA SECUENCIAL DE MARCADORES DE PROLIFERACIÓN Y APOPTOSIS DURANTE EL TRATAMIENTO DE LA PSORIASIS CON ANTI-FACTOR DE NECROSIS TUMORAL α (INFLIXIMAB)

Resumen. *Introducción y objetivos.* La psoriasis es una enfermedad inflamatoria cutánea de naturaleza inmunológica mediada por citoquinas de tipo Th1. El tratamiento con anticuerpos anti-factor de necrosis tumoral a (TNF-a) (infliximab) ha proporcionado respuestas clínicas significativas; sin embargo, los mecanismos implicados en la curación no están bien aclarados. El objetivo del presente trabajo es evaluar las variaciones de la histología y en la expresión de marcadores de proliferación y apoptosis, en biopsias cutáneas secuenciales de pacientes con psoriasis tratados con in fliximab.

Material y métodos. Se estudiaron biopsias de piel (sana y lesionada) de 3 pacientes afectados de psoriasis generalizada moderada-grave (índice de área y gravedad de la soriasis [PASI]: 35 de media) tratados con infusiones por vía intravenosa de infliximab (5 mg/kg) en las semanas 0, 2 y 6. Las biopsias se realizaron en los días 0, 14 y 28, y fueron procesadas para estudio histológico convencional e inmunohistoquímico con marcadores de apoptosis –TP53, BCL-2 y anticaspasas 3 y 8– y de proliferación celular –Ki67–.

Resultados. El tratamiento con infliximab se asoció con una significativa mejoría clínica en los 3 pacientes (PASI medio: 21,6 a los 14 días y 13,9 a las 6 semanas), que se correlacionó

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con la desaparición progresiva de las lesiones histológicas, con disminución de la proliferación epidérmica. Sin embargo, no observamos imágenes de apoptosis ni obtuvimos positividad con los anticuerpos anticaspasas. La expresión de TP53 disminuyó a las2 semanas del inicio del tratamiento, siendo similar a la piel normal a los 2 8 días.

Conclusiones. La respuesta clínica e histológica de la psoriasis con infliximab no se asoció a un incremento significativo en los marcadores de apoptosis evaluados.

Palabras clave: psoriasis, tumor necrosis factor a, histology, immunohistochemistry, apoptosis.

Introduction

Psoriasis is an autoimmune inflammatory skin disease whose etiopathogenic mechanisms are not well understood.[1,2] Serum concentrations of tumor necrosis factor (TNF)-α, which is the main cytokine implicated in the pathogenesis of psoriasis,[3] are correlated with the level of psoriatic activity. Treatment with anti-TNF-α antibodies (infliximab) has produced significant clinical response in patients with moderate to severe psoriasis.[4-5] Nonetheless, the precise mechanisms that underpin clinical cure are not entirely clear. By blocking TNF-α, infliximab acts on T-cell activation and proliferation. However, it is also possible that infliximab alters keratinocyte susceptibility to apoptosis, it being suggested that keratinocytes in the psoriatic epidermis have an abnormal resistance to programmed cell death.[6] This resistance may contribute to the epidermal hyperplasia (regular acanthosis) that is characteristic of the histopathology of psoriasis.

The aim of our study was, for serial skin biopsies taken from patients with psoriasis before and after infliximab treatment, to evaluate changes in conventional histology parameters and in the expression of apoptosis markers (TP53, Bcl-2, anticaspase-3, and anticaspase-8) and a cell proliferation marker (Ki67), and determine their importance with regard to the therapeutic effects of infliximab.

Material and Methods

Patients

Our study included 3 patients—2 men (aged 41 and 58 years) and 1 woman (aged 27 years)—with moderate to severe generalized psoriasis who no longer responded to treatment with systemic drugs and phototherapy. The 3 patients were treated with infliximab in the dermatology department of the Hospital Universitario Virgen Macarena, Seville, Spain. Diagnosis of the disease had been made at least 10 years previously, the average psoriasis area and severity index (PASI) was 35, and psoriatic lesions covered at least 50% of the body surface.

The systemic treatment and phototherapy were suspended 1 month prior to commencing treatment with infliximab (Remicade, administered in 100 mg vials). Doses were 5 mg/kg, administered intravenously in weeks 0, 2 and 6 (induction dose), and thereafter every 8 weeks (maintenance dose). Patients gave their consent to both the treatment and the biopsies.

Biopsies

The skin lesions were punch-biopsied (0.6 cm) on day 0 (before treatment with infliximab), and on day 14 (before the infusion of week 2) and day 28 of treatment with infliximab. Skin unaffected by psoriasis was also biopsied on day 0. The samples were fixed in a 10% buffered formaldehyde solution and embedded in paraffin. Tissue sections (4 µm thick) were stained with hematoxylin–eosin following the standard procedure.

Immunohistochemical Study

Serial 4-µm sections of all the samples were immunohistochemically studied with the following antibodies: TP53 (prediluted, Biomedia, USA), Ki67 (MIB-1, prediluted, Novocastra), Bcl-2 (1:50, Dako), caspase-3 (1:200, Novocastra), and caspase-8 (1:200, Novocastra). The immunoperoxidase techniques (streptavidin–biotin complex) were implemented using an automated immunostaining system (Ventana, Arizona, USA). The tissue sections were stained with diluted hematoxylin, and, once masked in regard to patient and time of biopsy, were semiquantitatively microscopically assessed by 2 observers (C. Gómez Mateo and J.J. Ríos Martín). Lymph node tissue was used as a positive control for the markers used in the study.

The TP53 protein, called the "guardian of the genome," initiates the apoptosis mechanism. The caspases are key apoptotic signal transduction and execution proteins,

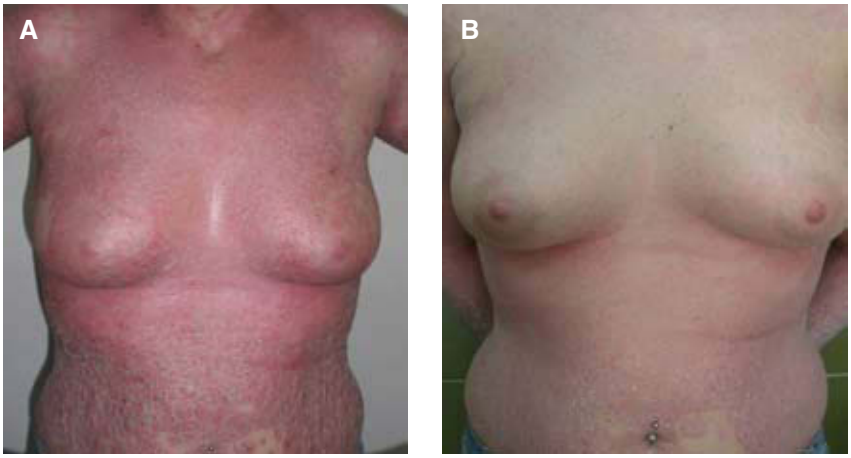

Figure 1. A. Patient with moderate to severe generalized psoriasis. B. Same patient on day 14 of treatment with infliximab.

Figure 2. Histology study of psoriatic skin before commencing treatment with infliximab. A. Histologically representative psoriasis vulgaris lesion (hematoxylin–eosin, ×200). B. Ki67 immunostaining, particularly evident in the basal epidermis (immunoperoxidase, ×200). C. TP53 immunostaining of over 10% of keratinocytes (immunoperoxidase, ×400).

whether as initiators (caspase-8) or effectors (caspase-3). Bcl-2 is an antiapoptotic protein.

Results

Histology Study

All 3 patients showed a significant improvement in their lesions (images A and B, Figure 1). In line with the clinical response, a gradual reduction was evident, between day 0 and day 28, in the histologic traits associated with psoriasis (regular epidermal hyperplasia, parakeratosis, lymphocyte and neutrophil exocytosis, superficial vascular dilatation, and dermal lymphohistiocytic inflammatory infiltrate at the surface of the perivascular site), as revealed by the skin biopsies (image A, Figures 2, 3, and 4). By day 28, only slight epidermal hyperplasia, with orthokeratosis and minimal dermal inflammatory infiltrate, was evident. Histologic images revealed no apoptosis in the epidermis (dyskeratosis) for any of the samples studied.

Immunostaining With Ki67

In the biopsies of lesioned skin performed before treatment (day 0), intensely positive Ki67 immunostaining of the basal keratinocytes marked out the entire basal lamina (image B, Figure 2). A slight reduction in staining was observed on day 14 (image B, Figure 3), and on day 28, epidermal proliferation was similar to that observed for normal skin (image B, Figure 4).

Immunostaining With TP53

Isolated nuclear-stained keratinocytes (1 to 2 keratinocytes per field [×20]) were observed in the biopsies of pretreated normal skin (day 0). In the biopsies of pretreated lesioned skin, TP53 expression was observed in over 10% of epidermal cells, particularly in the deeper-lying strata (image C, Figure 2). A significant reduction in staining was evident on day 14, with fewer than 5% keratinocytes resulting positive (image C, Figure 3). On day 28,

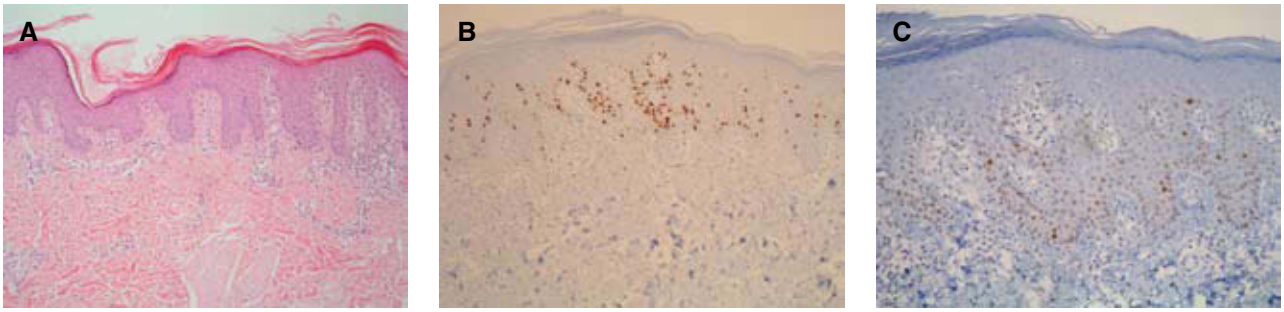

Figure 3. Histology study on day 14 of treatment with infliximab. A. Histology section showing a reduction in epidermal hyperplasia and inflammatory infiltrate, and also revealing parakeratosis (hematoxylin–eosin, ×100). B. Slightly reduced Ki67 immunostaining compared to pretreated skin (immunoperoxidase, ×100). C. Reduced TP53 immunostaining (less than 5% keratinocytes) compared to pretreated skin (immunoperoxidase, ×200).

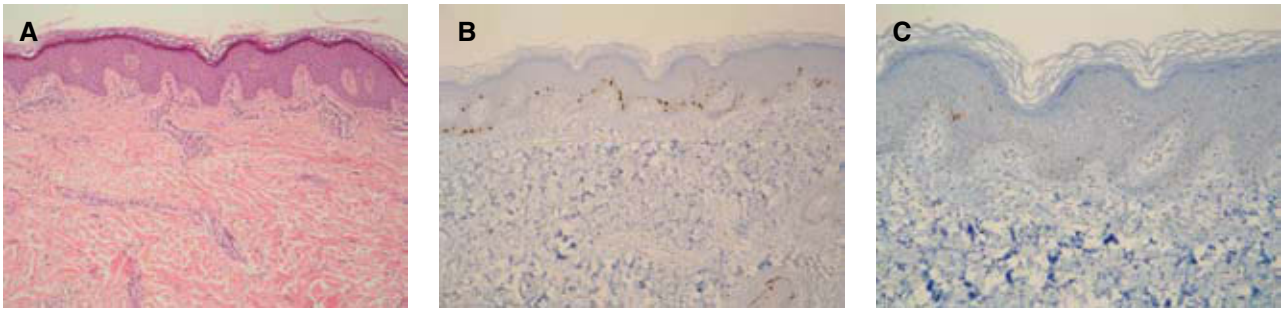

Figure 4. Histology study on day 28 of treatment with infliximab. A. No evidence of epidermal hyperplasia and normal keratinization (hematoxylin–eosin, ×100). B. Significantly reduced Ki67 immunostaining compared to pretreated skin (immunoperoxidase, ×100). C. TP53 immunostaining evident only in isolated keratinocytes (immunoperoxidase, ×400).

immunohistochemical expression of TP53 in lesioned skin was similar to that in normal skin (image C, Figure 4).

Immunostaining With Bcl-2

Bcl-2 immunostaining was negative in the keratinocytes of all the samples studied. Positive staining only occurred in melanocyte cytoplasms and lymphoid cells.

Immunostaining With Caspase-3 and Caspase-8

No immunostaining resulted for the anticaspase-3 and anticaspase-8 antibodies in any of the samples studied, whether of lesioned or unaffected skin.

Discussion

Treatment with anti-TNF-α has been demonstrated to be beneficial in patients with moderate to severe psoriasis.[4-5] We were able to report a clinical improvement in lesions and a reduction of 38% in the average PASI score in our patients 14 days after the first infliximab infusion; following the second infusion (week 2), the average PASI score had fallen by 60.28%. Response to treatment was thus very rapid. After the third infusion (week 6), 2 patients demonstrated a complete response and blanching of their lesions. Clinical lesions improved in parallel with the serial histology study.

The morphological findings were nonspecific for the samples obtained 28 days after the first infusion; only slight superficial perivascular lymphocytic infiltrate and mild irregular epidermal hyperplasia with orthokeratosis were observed. Thus, blocking soluble TNF-α produced a reduction in skin inflammation, and also reverted keratinocyte maturation processes to normal levels.

One of the mechanisms implicated in the pathogenesis of psoriasis is keratinocyte apoptosis suppression. It has recently been demonstrated that apoptosis-inhibiting proteins participate in this mechanism: survivin, which inhibits apoptosis mediated by the caspase pathway, and nuclear factor-κB, a transcription factor that regulates a number of genes implicated in apoptosis.[6] Furthermore, keratinocytes in the psoriatic epidermis seem to have an abnormal resistance to programmed cell death,[7] and other studies have demonstrated aberrant expression of apoptosis-related molecules.[8-10] TP53 expression in psoriatic skin is therefore probably a physiological consequence of epithelial hyperproliferation,[11,12] rather than a consequence of enhanced apoptosis.

Leaving aside antiinflammatory effects, it has been suggested by Krüger-Krasagakis et al[13] that the rapid response to infliximab is due to increased epidermal keratinocyte apoptosis, as demonstrated by immunohistochemical and electron-microscopy evaluations of TP53 expression. In our study, the biopsies of pretreatment lesions showed intense TP53 staining of the basal keratinocytes that reduced after 14 days, probably in association with an infliximab-induced reduction in cell proliferation. Nonetheless, immunostaining on day 14 was significantly greater than that observed in the biopsies of normal skin and in the biopsies performed on day 28. It is thus likely that the keratinocyte apoptosis mechanism is implicated in the clinical and histologic psoriatic response to infliximab, although, in our patients, there was no associated significant increase in TP53 immunostaining, nor did histologic images show cells in apoptosis. It is possible that initiation of this mechanism is an early event in lesion repair, as Krüger-Krasagakis et al[13] demonstrated an increase in TP53 expression in keratinocytes in biopsy samples obtained 5 days after commencing treatment. It would seem then, that this mechanism is not implicated in the caspase pathway, as demonstrated by these same authors for caspase-3.

The Ki67 immunostaining results indicate that infliximab reduces epidermal cell proliferation, and to a significant degree after 28 days. A similar conclusion was drawn by El-Domyati et al[14] in regard to their study of psoriatic lesion response to topical treatment with calcipotriol (a vitamin D3 analogue). It was suggested that calcipotriol inhibited cell proliferation by inducing apoptosis via a TP53-independent pathway (eg, the Fas-Fas ligand system or other members of the Bcl-2 family) through a mechanism similar to that for infliximab. In our study, however, we found no evidence of apoptotic bodies in the psoriatic epidermis, either before or after treatment.

Bcl-2 expression in psoriatic lesions does not appear to be related to proliferation or resistance to apoptosis. However, the results reported for this antibody are contradictory. Some studies have demonstrated immunohistochemical Bcl-2 expression in basal keratinocytes in normal skin but not in psoriatic skin,[15] whereas other studies have reported a reduction in Bcl-2 messenger RNA in psoriatic compared to normal skin.[8] Our results for Bcl-2 are similar to those described by Wrone-Smith et al[16]: there was no Bcl-2 expression in either normal or diseased epidermis. We did, however, observe Bcl-2 staining in the dermal and intraepidermal lymphocytes in the psoriatic lesion samples and in the melanocytes.

In conclusion, and despite the limitations implied by a descriptive study of 3 cases, it seems clear that psoriasis treated with infliximab normalizes keratinocyte differentiation, keratinocyte proliferation, and vascular dilatation, and reduces dermal inflammatory infiltrate density—all of which lead to rapid clinical improvement in psoriatic lesions. Nevertheless, in the light of our own results and those reported in the literature, what still remains far from clear is the role played by apoptosis in the mechanism of action of infliximab.

Conflicts of Interest
The authors declare no conflicts of interest.

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