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In situ Expression and Serum Levels of Tumour Necrosis Factor Alpha Receptors in Patients with Lichen Planus

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In lichen planus (LP), an inflammatory skin disease of unknown origin, adhesion molecules and their ligands combined with the local and systemic release of various cytokines are fundamental in regulating inflammation. Therefore, we investigated the expression of tumour necrosis factor alpha receptor (TNF-R) I and II in lesional skin of 15 patients suffering from acute eruptive LP by means of immunohistochemistry. In addition, the serum levels of their soluble forms (sTNF-R) were measured by enzyme-linked immunosorbent assay (ELISA), compared with those of healthy volunteers ($n=10$) and correlated with the *in situ* inflammatory response. In contrast to healthy controls, LP patients showed significantly increased serum levels of sTNF-RI as well as sTNF-RII ($p<0.02$). These enhanced serum titres were correlated with a prominent expression of TNF-RI on lesional keratinocytes (basal > suprabasal) and of both receptors on skin-infiltrating lymphocytes. Our data suggest an important role of the TNF ligand/receptor interactions in the induction and/or perpetuation of the pathogenetical and apoptotic events in LP. ***Key words: inflammatory dermatosis; cytokine receptor; immunohistochemistry; ELISA.***

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Lichen planus (LP) (1) is an inflammatory skin disease, characterized by a dense infiltrate of HLA-DR$^+$ CD25$^+$ LFA-1$^+$ CD3$^+$ CD8$^+$ TCR$\gamma\delta^+$ T-lymphocytes at the dermo-epidermal junction, combined with a local and systemic release of various cytokines both in the skin and in the serum (2–4). The pathogenetical relevance of cytokines in inflammatory skin diseases is well recognized (5). Tumour necrosis factor alpha (TNF-α) and interferon-γ (IFN-γ) induce HLA-DR, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on the surface of keratinocytes and/or endothelial cells in inflamed tissue (6) and are able to initiate apoptotic cell death (7). To gain more insight into the pathogenesis of LP, we investigated the expression of TNF-RI and -RII in lesional skin and the serum levels of their soluble forms (sTNF-R) in patients suffering from acute, eruptive onsets of the disease.

MATERIALS AND METHODS

Patients

Four-millimeter punch biopsies from lesional skin of 15 LP patients and 10 cm^3 of peripheral blood were obtained from each of the 15 patients with acute, eruptive LP and 10 healthy volunteers. Skin specimens from healthy probands ($n=10$) served as controls. The clinical diagnosis was confirmed in each case by histopathology (8). None of the patients or healthy control subjects had had common cold or other infectious diseases for at least 3 weeks before blood and skin samples were obtained, and none of the patients received immunosuppressive therapy.

Skin specimens and immunohistochemistry

Frozen skin specimens were prepared for sectioning by embedding in OCT compound (Lab-Tek, Naperville, Ill, U.S.A.), sectioned at 4 µm with a cryostat at −20°C and placed on poly-L-lysine-coated slides (MW > 300,000, 50 µg/ml, Sigma, St. Louis, MO, U.S.A.). Immunohistochemistry was performed on air-dried, aceton-fixed serial frozen sections of each sample. Monoclonal antibodies (moAbs) specific for TNF-receptors were kindly provided by M. Brockhaus (Hoffmann-LaRoche AG, Basel, Switzerland): htr-9 (specific for human 55kD TNF-R, TNF-RI, CD120a, working concentration 100 µg/ml) and utr-1 (specific for human 75kD TNF-R, TNF-RII, CD120b, working concentration 100 µg/ml) (9). Immunohistochemical stainings were performed as previously described (10), using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) complex technique and hexazotated new fuchsine as chromogenic substrate. Cells stained immunohistochemically in serial sections at 5 planes of each skin biopsy were investigated by two independent observers. Percentage of positive cells was calculated semiquantitatively as stained cells in proportion to all cells of a distinct cell subset.

Immunofluorescence

In single staining indirect immunofluorescence (IIF) tests, the above mentioned moAbs against TNF-Rs were incubated for 30 min at room temperature, appropriately diluted in phosphate-buffered saline (PBS) (pH = 7.2). MoAbs were detected by a Fab_2 fragment of tetramethyl-rhodamine-isothiocyanate (TRITC)-labelled goat anti-mouse immunoglobulin (Jackson Immuno Research, West Grove, PA, U.S.A., code: 115–026–062). In double staining immunofluorescence sections were first incubated (30 min, room temperature) with a purified rabbit IgG against human CD3 (Dakopatts, Glostrup, Denmark, code: A452), followed by a FITC-conjugated Fab_2 fragment of swine anti-rabbit immunoglobulin (Dakopatts, code: F0054). Subsequently, moAb against TNF-R was incubated for 30 min and detected by a Fab_2 fragment of TRITC-labelled goat anti-mouse immunoglobulin. After each incubation the sections were thoroughly washed in PBS (pH = 7.2) for 30 min and after a final wash for 90 min mounted in buffered glycerol.

Control stainings

Omission of first step antibody and staining with a non-sense moAb of the same immunoglobulin isotype served as negative controls.

Enzyme-linked immunosorbent assay (ELISA)

Soluble cytokine-receptor serum levels were determined by immunoassays specific for human TNF-RI (DRT100, R&D Systems, 7.8–500 pg/mL, sensitivity 1 pg/mL) and TNF-RII (DRT200, R&D Systems, 7.8–500 pg/mL, sensitivity 0.5 pg/mL).

Statistical analysis

Differences in means between normal and LP sTNF-R values were analyzed using Student's *t*-test for paired data.

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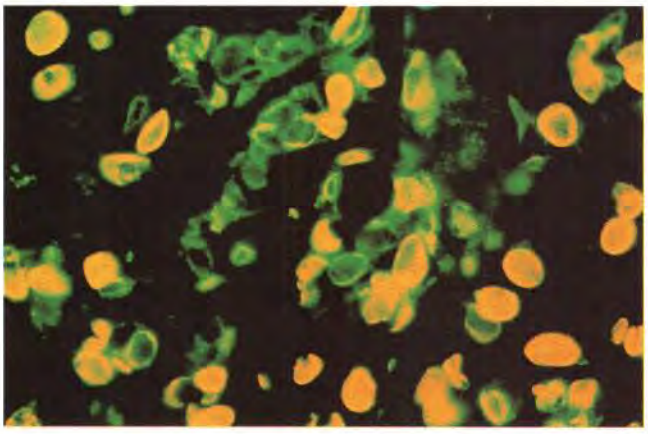

Fig. 1. Immunofluorescence double staining in a lichen planus lesion using specific antibodies against CD3 (FITC, green) and TNF-RI (TRITC-conjugated secondary antibody, red): co-expression in mononuclear infiltrating cells demonstrated by yellow staining (original magnification ×400).

RESULTS

Expression pattern of mononuclear infiltrating cells

The dense subepidermal, band-like mononuclear inflammatory infiltrate mainly consisted of $CD3^+$ T-lymphocytes, which expressed TNF-RI in 60–80% (Fig. 1) and TNF-RII in about 40–60%.

Expression pattern of keratinocytes

Whereas TNF-RII could not be detected on keratinocytes in LP lesions and healthy skin, TNF-RI was strongly expressed on lesional keratinocytes with adjacent inflammatory infiltration (Fig. 2). In comparison to the upper epidermal cell layers TNF-RI expression on keratinocytes was more pronounced in the basal cell compartment. In contrast, keratinocytes of normal skin, derived from healthy volunteers, showed a constitutive expression of TNF-RI throughout all epidermal layers.

Serum titres of sTNF-RI and -RII

The serum levels of sTNF-RI and -RII detected in LP patients and healthy controls are presented in Table I. The serum levels of both receptors were statistically significantly higher in the patients than in healthy controls ($p < 0.02$).

DISCUSSION

Cytokines are low-molecular-weight (glyco)proteins that are transiently produced and exert their biologic activities via specific cell-surface receptors. Cytokines regulate cell metabolism and functions; in addition, they exhibit immunoregulatory activities through a complex cytokine network consisting of paracrine and autocrine systems (11, 12).

TNF-α is a pleiotropic cytokine, generated from macrophages, activated T-lymphocytes and keratinocytes, and believed to play either a beneficial (protective) or a deleterious (pathologic) role in inflammation and immunological reactions, depending on timing, target cell, and magnitude of the

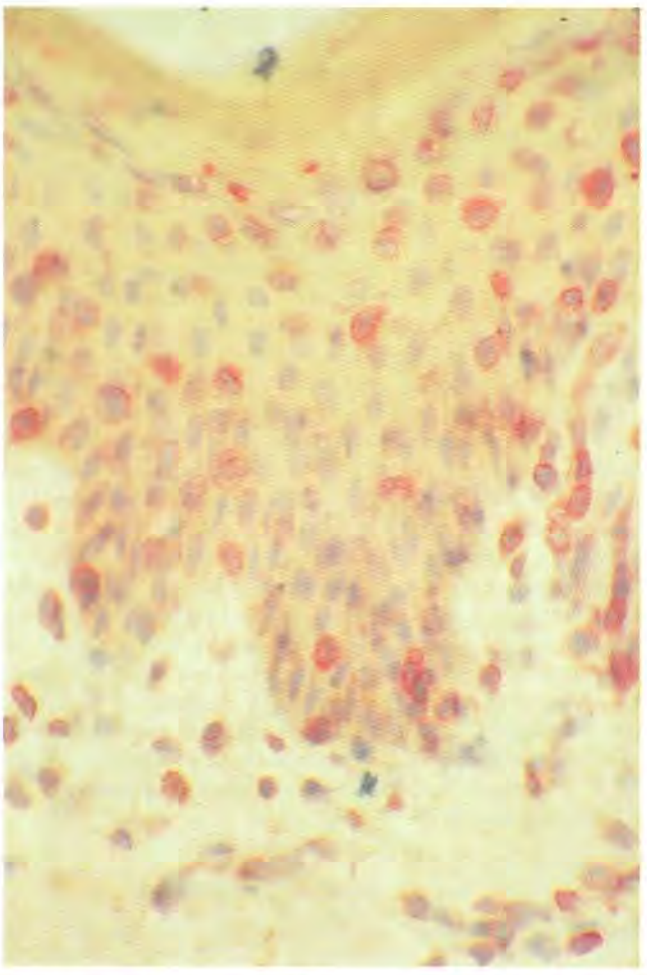

Fig. 2. TNF-RI-positive lesional keratinocytes and mononuclear cells in lichen planus (original magnification ×250).

Table I. *sTNF-R serum levels (mean ± SE; pg/mL) in patients with LP and in healthy controls*

Patients	*n*	sTNF-RI	sTNF-RII
LP	15	1293 ± 140 ($p < 0.02$)	3055 ± 338 ($p < 0.02$)
Controls	10	844 ± 110	1926 ± 248

inflammatory reaction (13). High-affinity membrane receptors for TNF-α exist on many tissues, including liver, muscle, gut, kidney, lung and skin. The exact cellular location(s) of these receptors in the skin is still uncertain, although they have been detected on epidermal and endothelial cells as well as on dermal fibroblasts (14–16). Two distinct types of receptors have been cloned and sequenced that specifically bind TNF-α: a 55kD form designated TNF-RI and a 75 kD form designated TNF-RII (9, 17). It has been shown that TNF-RI plays a decisive role in the host's defence against microorganisms and their pathogenic factors, while TNF-α binding to TNF-RII plays a role in the induction of tissue necrosis (18).

Recent clinical and immunopathological studies suggest a T-cell-mediated autoimmune reaction involved in the pathogenesis of LP (4, 19). Among these are the predominance and close vicinity of activated, $HLA\text{-}DR^+$ $CD25^+$ $LFA\text{-}1^+$ $CD8^+$

TCR$\gamma\delta^+$ T-lymphocytes and macrophages/Langerhans' cells in the dermo-epidermal inflammatory infiltrate (20), combined with a local and systemic release of various cytokines (e.g. TNF-α, IFN-γ) both in the skin and in the serum (21–23), and the liquefying degeneration of basal keratinocytes with damage to the basement membrane (3, 8). In the course of examining keratinocyte-lymphocyte interactions *in vitro*, Nickoloff et al. (24) reported prominent adherence by both allogeneic and autologous T-lymphocytes and monocytes to cultured keratinocytes after pretreatment of the keratinocytes with IFN-γ. The binding of these mononuclear cells to IFN-γ-treated keratinocytes involves the lymphocyte function-associated antigen-1 (LFA-1) molecule on T-cells and the ICAM-1 induced by IFN-γ or TNF-α on keratinocytes (6). Our present findings concerning the elevated serum levels of both sTNF-R with a prominent expression of both TNF receptors on skin-infiltrating lymphocytes and lesional keratinocytes (TNF-RI), especially on the basal layers of the epidermis in LP, indicate that TNF-α and IFN-γ play, synergistically (25), a critical role in the induction and perpetuation of the pathogenetical, probably apoptotic (26), events in LP. From this point of view it is not surprising that certain therapeutic modalities, such as PUVA, cyclosporin A and aromatic retinoids, known to disrupt mechanisms by which keratinocytes receive the signals to synthesize MHC class II antigens and various adhesion molecules (processes mediated by TNF-α and IFN-γ), are successful in the treatment of LP. In addition, serum levels of TNF-R might be reliable and reproducible markers of the disease activity in patients with LP.

Since members of the TNF-R family can mediate apoptosis (7), it is conceivable that TNF-R^+ $CD8^+$ cytotoxic T-lymphocytes, in concert with soluble mediators such as TNF-α, may induce apoptotic cell death in LP. Whether the increased expression of TNF-R in lesional keratinocytes and infiltrating mononuclear inflammatory cells, as shown in our patients, correlates with apoptosis of epidermal cells or T-lymphocytes (27) in the skin lesions of LP patients remains to be elucidated.

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