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# Endothelin-1 Levels Are Increased in Sera and Lesional Skin Extracts of Psoriatic Patients and Correlate with Disease Severity

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**Endothelins (ETs), in addition to their systemical activities, exert important functions at the skin level, such as increase of keratinocyte proliferation, neo-angiogenesis and leukocyte chemotaxis, which are among the main characteristics of psoriasis. To assess a possible ET-1 involvement in plaque-type psoriasis, ET-1 determinations were carried out in 15 sera and 8 lesional and non-lesional biopsy skin extracts from psoriatic patients and in 15 sera and 5 biopsy skin extracts from healthy volunteers, sex- and age-matched, using commercially available ELISA kits.**

**A statistical analysis of the results showed that ET-1 levels were increased in sera of psoriatic patients, as compared to normal subjects ($p=0.04$). In addition, there was a significant correlation between both serum ($r=0.60$, $p=0.02$) and lesional skin ($r=0.80$, $p=0.03$) ET-1 values versus the Psoriasis Area and Severity Index scores.**

**Significant increases of the lesional versus the non-lesional ($p=0.01$) and versus the normal ($p=0.04$) ET-1 skin extract values were observed, together with a significant correlation between lesional and non-lesional ET-1 skin levels ($r=0.79$, $p=0.03$).**

**These findings were also confirmed at the mRNA level, using RT-PCR analysis, where increased ET-1 mRNA levels, densitometrically measured, were found in the lesional samples versus non-lesional and normal skin.**

**Since interleukin-8 is involved in psoriasis and shares some biological properties with ET-1, we further evaluated the levels of this cytokine in skin extracts. The behaviour of interleukin-8 paralleled that of ET-1, and a significant correlation between these two molecules was observed in the lesional skin ($r=0.76$, $p=0.05$).**

**Taken together, these data stress that, as previously described for interleukin-8, ET-1 may be involved in inflammatory processes associated with psoriasis. *Key words: IL-8; PASI score; mRNA.***

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Endothelins (ETs) are members of a 21 amino acid peptide family, represented by ET-1, ET-2 and ET-3 and having profound cardiovascular, mitogenic and neuroregulatory actions (1, 2). These peptides are secreted by different cell types, including macrophages (3), monocytes (3), astrocytes (2, 4), hepatocytes (2), kidney mesangial cells (2), vascular smooth muscle cells (2), endothelial cells (2) and various tumour cells (5). Furthermore, ET-1 is secreted by human keratinocytes and is involved in the regulation of melanocyte proliferation and pigmentation (6).

Recently, it has been reported that primary human keratinocytes express ET-A receptors and produce ET-1, which stimulates growth responses, demonstrating that endogenous ET-1 is one of the most important autocrine growth factors for keratinocytes (7). Moreover, ET-1 could be involved in inflammatory mechanisms, as shown by their ability to stimulate neutrophil chemotaxis (8).

In normal human skin, immunohistochemical studies have shown that ET-1 is mainly expressed in the blood vessel walls, including the capillaries of dermal papillae. Autoradiographic investigations revealed a $^{125}$I-labelled ET-1 binding over capillaries and larger blood vessels, as well as hair follicles and sweat glands, indicating the presence of specific receptors (9).

The strategical localization of ET-1 and its receptors, at the vascular skin level, underlines the importance of this vasoconstrictor peptide in the maintenance of core body temperature (10).

In addition, in patients affected with systemic scleroderma, the increased ET-1 mitogenic activity observed on dermal fibroblasts and the increased ET-1 levels determined in plasma samples are in agreement with a role of this molecule in dermal fibrosis (11).

Taken together, all these properties suggest that ET-1 could play a role both in the maintenance of normal cutaneous homeostasis and in the pathophysiology of cutaneous diseases, such as psoriasis, characterized by increased keratinocyte proliferation, neo-angiogenesis and enhanced leukocyte chemotaxis (12).

In the present study, we evaluated the serum ET-1 levels together with ET-1 amounts and ET-1 mRNA expression in skin extracts of lesional and non-lesional biopsy samples from psoriatic patients, compared to skin extracts from normal individuals.

In addition, since interleukin-8 (IL-8) has been reported to be involved in the pathogenesis of psoriasis (13) and to share some biological functions with ET-1, such as keratinocyte growth, vascular proliferation and leukocyte chemotaxis (14, 15), we also evaluated the levels of this cytokine in extracts of lesional and non-lesional psoriatic skin to verify possible quantitative correlations.

The findings shown in this work may be useful to highlight a possible ET-1 involvement in the inflammatory processes associated with plaque-type psoriasis.

## MATERIALS AND METHODS

Serum samples were obtained from 15 patients affected with active plaque-type psoriasis (4 males and 11 females, median age: 53 years, range 31–75).

The disease severity was evaluated by means of the Psoriasis Area

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and Severity Index (PASI). In our patients the median PASI score was 12.4, ranging from 6.3 to 31.7.

None of the patients had received topical or systemic antipsoriatic treatments for at least 10 days before enrollment. Cyclosporin A had not been used in these patients during the previous cycle of therapy, excluding a possible induction of ET synthesis by this drug (15).

Other concomitant diseases were excluded by means of a complete clinical and laboratory examination. After blood collection, sera were stored at −80°C until used.

In 8 (4 males and 4 females; median age: 48 years 34–70; median PASI score: 14.5, range 6.3–31.7) of the 15 patients, 6-mm diagnostic punch biopsies were also performed, under local anaesthesia with 0.2–0.3 ml of 2% lidocaine, without epinephrine, from lesional and non-lesional skin (at least 5 cm away from the lesion). These samples were stored at −80°C until homogenization procedure. Briefly, the skin samples were homogenized in 1 ml of RPMI 1640 (Gibco BRL, Paisley UK) by means of a glass homogenizer with a motor-driven ground glass pestle and then centrifuged at 10,000 rpm for 15 min, at 4°C. The extracts were passed through 0.45-mm filters and immediately stored at −80°C.

As a control, serum samples were obtained from 15 healthy volunteers (5 males and 10 females, median age 50 years, range 29–70). In 5 of the 15 controls, 6-mm punch biopsies were also performed at the volar surface of the forearm for the skin extraction procedures.

For ET-1 and IL-8 determinations two commercially available ELISA kits (purchased from R&D: Human IL-8 and Human Endothelin-1 immunoassays; R&D Systems, Inc., Minneapolis, USA) were used according to the manufacturer's instruction.

For serum ET-1 measurements, 0.75 ml of an extraction solution including acetone:1 M HCl:water (40:1:5) were added to 0.5 ml of the samples, and the mixture was then centrifuged for 20 min at 3,000 rpm in a refrigerated centrifuge, at 4°C. Supernatants were decanted into polypropylene tubes and dried in a centrifugal evaporator (Speedvac) for 5 h at 37°C. The pellets were reconstituted with 0.125 ml of sample diluent immediately before ET-1 measurements.

The sample total protein (TP) content was measured using the Coomassie Protein Assay Reagent (Pierce Chemical, Rockford, USA) according to the manufacturer's recommendations.

For RT-PCR analysis, total RNA was extracted and purified from 3-mm punch biopsies by a commercial kit: RNAzol (Biotech Lab. Inc., Houston, Texas), using the guanidinium thiocyanate method (17). Two control subjects and three psoriatic patients were arbitrarily selected without knowing their serum and skin ET-1 levels. Due to the presence of degraded mRNA, the results of only one control and 2 patients are presented.

One microgram of RNA was reversely transcribed to cDNA and amplified by the RNA PCR kit (Perkin Elmer Co., Norwalk, CT), according to the manufacturer's instructions, with specific primers for the ET-1 mRNA; the upstream primer, (5′-TGCTCCT GCTCGTCCCTGATGGATAAAGAG-3′) and the downstream primer, (5′-GGTCACATAACGCTCTCTGGAGGGCTT-3′) as reported by Pekonen et al. (18). Briefly, reverse transcription was carried out at 42°C for 15 min in presence of downstream primers. The reaction was stopped by incubation at 99°C for 5 min and chilled in ice. The cDNA was amplified with 2.5 U Amplitaq (Roche, USA) for 30 cycles in presence of up- and downstream primers for ET-1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were included as an internal control. In all experiments, two control reactions, one containing no mRNA and the other containing no reverse transcriptase, were included. The amplified products were analyzed in a 2% Nu-Sieve agarose gel. Quantification of amplified products was achieved by scanning densitometry of the bands in ethidium bromide-stained gel and expressed as relative levels of specific mRNAs normalized to those of GAPDH.

*Statistical analysis*

Due to the unknown data distribution types, the results were expressed as median, minimum and maximum. Accordingly, the following non-parametric tests were used: the Wilcoxon rank test, the Mann–Whitney test and, finally, the Spearman's rank correlation test.

## RESULTS

*ET-1 in sera*

To compare serum ET-1 levels of psoriatic and normal subjects, 15 serum samples from the patients and 15 from the controls were assayed using ELISA techniques. The serum median ET-1 levels were increased in the patients (median 0.9 pg/ml; minimum 0.4 pg/ml; maximum 1.7 pg/ml) as compared with the control group (median 0.6 pg/ml; minimum under detection limit; maximum 0.8 pg/ml) ($p = 0.04$).

In addition, to verify whether the patient ET-1 values were related to the disease severity, a correlation test was performed on the patients' data. As shown in Fig. 1A, a significant correlation between serum ET-1 levels and PASI scores was observed ($r = 0.60$; $p = 0.02$).

*ET-1 in skin extracts*

Other experiments were undertaken to investigate whether the difference observed at the systemic level could also be revealed

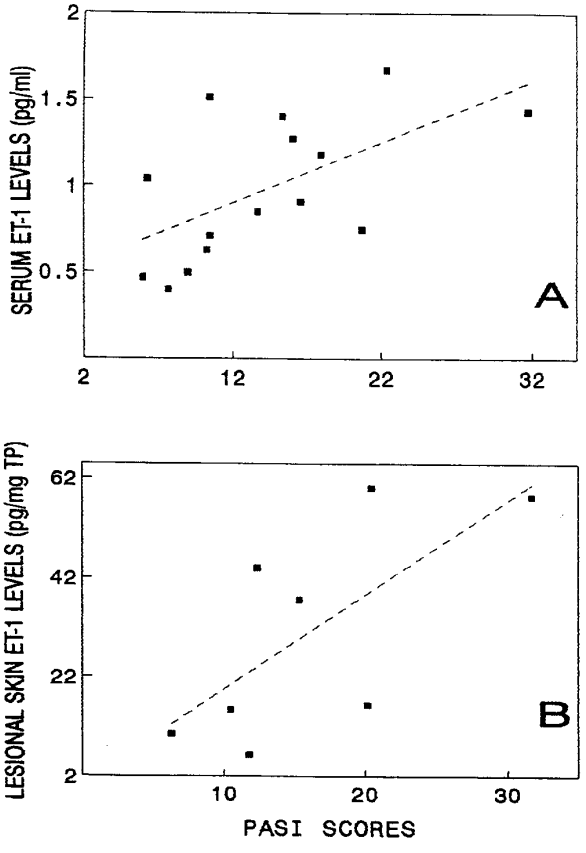


*Fig. 1.* Correlations between the PASI scores and (A) the serum ET-1 levels of 15 psoriatic patients ($r = 0.60$, $p < 0.02$); (B) the lesional skin ET-1 levels of 8 psoriatic patients ($r = 0.79$, $p < 0.03$). *TP* = total protein.

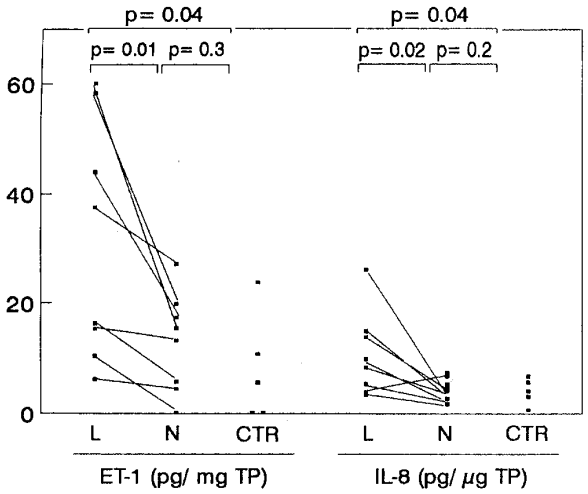


*Fig. 2.* Distribution of the ET-1 and IL-8 levels evaluated in lesional (*L*), non-lesional (*N*) and control (*CTR*) skin extracts. Significances of comparisons are shown as the top. *TP* = total protein.

at the local level, comparing 8 lesional and 8 non-lesional skin extracts with 5 extracts from controls.

The analysis of the results showed a significant increase of ET-1 levels in lesional psoriatic skin (median 27.0 pg/mg TP, minimum 6.3 pg/mg TP; maximum 60.0 pg/mg TP) as compared with either non-lesional skin (median 14.5 pg/mg TP; minimum under detection limit; maximum 28.0 pg/mg TP; $p = 0.01$) or normal skin (median 5.7 pg/mg TP, minimum under detection limit; maximum 23 pg/mg TP; $p = 0.04$). No statistically significant difference was found between non-lesional and normal skin ($p = 0.3$) (Fig. 2).

Furthermore, to explore the possibility that a quantitative relationship could exist between the lesional and the non-lesional skin of the patients, a correlation test between the corresponding ET-1 concentrations was performed: a significant regression coefficient was observed ($r = 0.79$, $p = 0.03$).

As previously reported for serum ET-1 amounts, a correlation between lesional skin extract ET-1 levels and PASI scores was also noted ($r = 0.80$; $p = 0.03$) (Fig. 1B).

No relationships were found in patients or controls, either at the systemic or the local level, between ET-1 concentrations and: age, sex, smoking history, and physical activity (data not shown).

### *ET-1 mRNA in skin extracts*

To verify and confirm results obtained by ET-1 ELISA from skin extracts, mRNA from small biopsies was extracted and specific messengers for ET-1 were reversely transcribed and amplified by RT-PCR. A single DNA fragment of the expected size (462 bp) was amplified in the skin samples. Fig. 3A shows the amplification signal of ET-1 mRNA from 2 psoriatic patients and one healthy volunteer. The specificity of this band was confirmed by southern blot analysis with a $^{32}$P-labelled internal probe (data not shown).

The densitometric analysis of the bands showed an increased expression of mRNA for ET-1 in lesional skin samples of psoriatic patients, compared with non lesional and with normal skin (Fig. 3B).

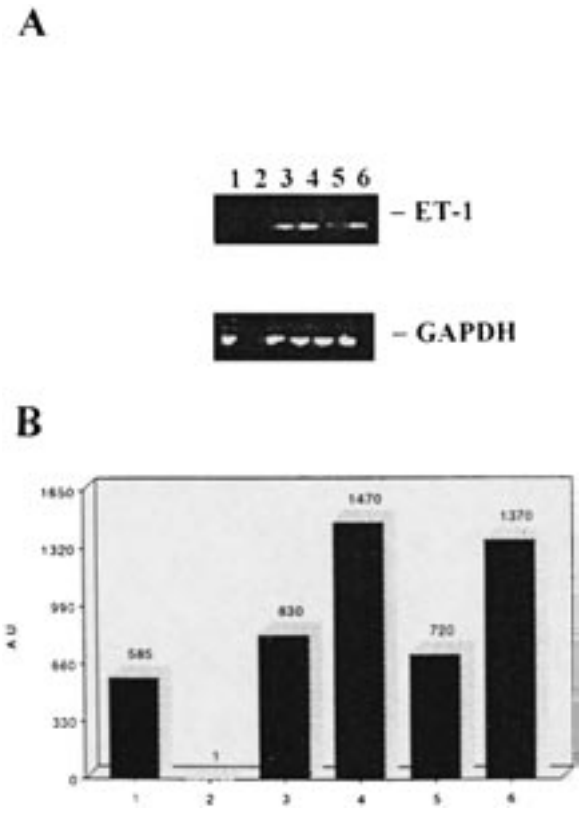


*Fig. 3.* Amplification products of ET-1 and GAPDH mRNAs in five representative skin samples. *Lane 1*; control of normal skin sample from healthy volunteer, *lane 2*; control sample with no mRNA, *lanes 3–4 and 5–6*; non-lesional and lesional skin samples of 2 psoriatic patients. The predicted sizes for the amplification products were 462 bp for ET-1 and 535 bp for GAPDH. (A) Ethidium bromide-stained gel of the amplified products. (B) densitometric analysis expressed as relative levels of the specific mRNA normalized to those of GAPDG. *A.U.* = arbitrary units.

These results correlated perfectly with those from ELISA experiments, confirming that psoriatic skin expressed higher levels of specific mRNA for ET-1.

### *IL-8 in skin extracts*

IL-8 determinations are reported only for skin extracts, due to the fact that serum levels were mainly under detection limits.

The skin extract data (Fig. 2) confirmed that IL-8 is increased in lesional psoriatic skin (median 8.1 pg/μg TP; minimum 3.5 pg/μg tp; maximum 26.2 pg/μg TP) as compared to non-lesional (median 5 pg/μg TP; minimum 1.9 pg/μg TP; maximum 7.1 pg/μg TP; $p = 0.02$) and normal skin (median 4.5 pg/μg TP, minimum 0.8 pg/μg TP; maximum 5.9 pg/μg TP: $p = 0.04$). This is in agreement with previous findings published in the literature (13, 15).

Interestingly, at the lesional level, we could observe a significant correlation between ET-1 and IL-8 levels ($r = 0.76$, $p = 0.05$) (Fig. 4). As expected, the above-mentioned results showed that IL-8 amounts were significantly correlated with the PASI scores ($r = 0.78$, $p = 0.04$).

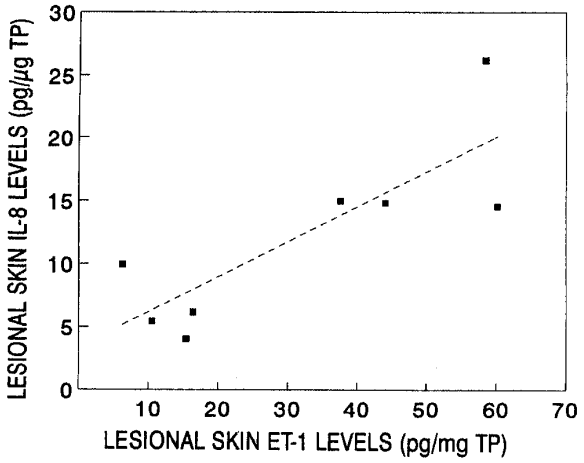


*Fig. 4.* Correlation of the ET-1 and IL-8 levels observed in lesional skin extracts ($r=0.76$, $p=0.05$). *TP*=total protein.

## DISCUSSION

Previous reports have revealed a significant increase in ET-1 and ET-2 plasma levels in psoriatic patients, in comparison with the controls (19, 20).

Our study, showing increased serum ET-1 concentrations in psoriatic patients, also found a direct correlation between serum ET-1 levels and PASI scores, suggesting that ET-1 is linked to the PASI score, principally representing an index of the disease extension. Therefore, it is possible that serum ET-1 measurements can be useful in monitoring psoriasis.

To evaluate the possibility that the increased serum ET-1 concentrations could depend on lesional skin amounts, the present paper analyzed the ET-1 levels of lesional, non-lesional and normal skin. To standardize possible differences due to the biopsy variations, the data were expressed as pg/TP.

At the local level, we found that skin extracts of lesional psoriatic skin contained higher ET-1 concentrations as compared to non-lesional and normal skin, indicating that the highest levels of ET-1 were associated with the psoriatic plaque. The uninvolved skin did not show significant differences in comparison with the skin of normal individuals, although an evident correlation could be observed between ET-1 amounts measured in lesional and non-lesional skin of the same patients, suggesting a possible participation of the whole skin in the disease (21–23).

The data obtained at the protein level were confirmed at the mRNA level by RT-PCR analysis, performed in skin extracts. Thus, the same increasing concentration gradient of ET-1 is evident for normal, non-lesional and lesional skin.

As observed for serum ET-1 levels, skin extract ET-1 concentrations were also significantly correlated to the PASI scores, suggesting a direct relationship between the local ET-1 amounts and blood concentrations and between the latter and the disease severity score.

Since IL-8 is known to induce ET-1 synthesis in epithelial cells (24), and considering that IL-8 and ET-1 share several biological functions and that an involvement of IL-8 is already known in psoriasis, we measured the concentrations of this biological modulator in the same samples.

Interestingly, our results indicated that the lesional ET-1 behaviour paralleled that of IL-8. In fact, skin extract IL-8 was increased in the lesional skin and was significantly correlated to the PASI scores. No significant difference was found between normal and non-lesional skin. Finally, in accord with a possible ET-1 induction by IL-8, or alternatively a common induction by TNF-alpha (24), a significant correlation was observed between ET-1 and IL-8 amounts, at the lesional level.

Although this type of investigation does not provide information concerning the cellular source of this molecule, on the basis of the previous data, it is supposable that ET-1 mainly originates from endothelial cells, keratinocytes and mastcells.

Some speculations could be raised concerning the significance of the high ET-1 concentrations observed in involved areas of psoriatic skin.

In vitro studies clearly indicate that IL-8 induces ET-1 synthesis and that both act as mitogenic factors for the keratinocyte (6, 14, 24). In particular, the inhibition of the basal growth of keratinocytes in the presence of a specific antagonist for the ET receptors demonstrates that ET-1 is one of the most important factors involved in keratinocyte proliferation, which, in turn, could lead to abnormal proliferation and turnover of keratinocytes in psoriasis (6).

Since ET-1 is also mitogenic for endothelial and vascular smooth muscle cells (25), it could participate in the angiogenetic processes (26), reported to be one of the earliest changes seen in the psoriatic lesion (27), probably driven by IL-8 and other cytokines, in the context of a more complex network.

A controversial point might be the discrepancy between the strong vasoconstrictive property of ET-1 and the marked dilation of the papillary microvessels characteristic of psoriatic lesions and possibly due to the increased amounts of inducible nitric oxide synthase, recently reported (28). ET-1 increase may also be the result of endothelial cell activation (with subsequent ET-1 synthesis and release) and it is possible that ET-1, reflecting the activation of inflammatory processes, only represents the biological response directed to control vasodilation.

Interestingly, the mitogenic activity of this molecule both on keratinocyte and endothelium, together with its neoangiogenic properties, may contribute to maintain the psoriatic manifestations.

Finally, IL-8 and ET-1 molecules contribute to the leukocyte influx described in the lesional skin, due to their potent chemoattractant activities (8).

Although our findings are suggestive of a role of ET-1 in the psoriatic cytokine network, further investigations are in progress to better clarify its activity in the inflammatory phenomena associated with this dermatosis.

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