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Levels of Skin-Derived Antileukoproteinase (SKALP)/Elafin in Serum Correlate with Disease Activity During Treatment of Severe Psoriasis with Cyclosporin A

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The epidermal serine proteinase inhibitor SKALP (also known as elafin), directed against human leukocyte elastase and proteinase 3, is strongly induced in suprabasal keratinocytes during inflammation. The presence of SKALP/elafin in urine has been demonstrated for several inflammatory skin disorders, such as psoriasis, erythroderma, and erysipelas. In this study we investigated whether SKALP/elafin levels in serum and urine of psoriatic patients can be used as a marker for disease activity during treatment. Patients with severe chronic disabling psoriasis were treated with cyclosporin A, which resulted in a marked clinical improvement as measured with the PASI score. SKALP/elafin levels both in serum and urine were determined with an enzyme-linked immunosorbent assay (ELISA). Measurements were performed at the start of the cyclosporin A treatment, and after regular intervals up to 16 weeks. The results indicate that 1) SKALP/elafin determination in serum rather than in urine is the preferred method, because the decrease in serum SKALP levels during therapy is more pronounced and correlated better with the clinical course of the patients; 2) SKALP/elafin levels in serum decreased during cyclosporin A treatment (p < 0.05); and 3) SKALP/elafin levels in serum correlate with the PASI score (p < 0.01). We conclude that SKALP/elafin measurement in serum of patients with severe psoriasis provides a tool for monitoring disease activity. Keywords: ELISA, proteinase inhibitor, epidermis, J Invest Dermatol 104:189-193, 1995

We have recently shown that skin-derived antileukoproteinase (SKALP), a serine proteinase inhibitor virtually absent in normal epidermis, is induced in lesional psoriatic skin [1]. We found that SKALP, also known as elafin [2,3], is not specific for psoriasis, but can be considered as one of the markers of the regenerative differentiation program that is also found in other conditions such as wound healing [4,5] and several inflammatory skin diseases [6]. SKALP/elafin is a strong inhibitor of the leukocytic enzymes elastase [1] and proteinase-3 [7], and is putatively involved in the regulation of cutaneous inflammation. In previous studies SKALP/elafin was described biochemically [8] and characterized at the protein and DNA level [9]. We established the cellular source and localization in psoriatic epidermis [10], and we found that SKALP/elafin is differentially expressed in human epidermal tumors [11]. Recently we have been able to assign the SKALP/elafin gene to chromosome region 20q12-q13 [12]. The gene has been given the approved name of Protease Inhibitor, skin-derived (SKALP) and the symbol PI3 in the Genome Data Base of the HUGO nomenclature committee.

In psoriasis, SKALP/elafin is expressed in the upper spinous layers of lesional skin [10]. We showed that SKALP/elafin as expressed in cultured epidermal keratinocytes is translated as a 12.3-kDa protein. Cleavage of the signal peptide yields a 9.9-kDa protein that is the major form found in cultured cells, as was confirmed by purification and N-terminal amino acid sequencing [9]. The presence of a signal peptide suggests that SKALP/elafin is a secreted protein. We indeed found that significant amounts of biologically active SKALP/elafin can be found in the urine of psoriatic patients [13], a finding that was recently confirmed by others [14]. In urine of healthy individuals SKALP/elafin was not detectable. Apparently, once secreted in the epidermis, SKALP/elafin is able to reach the circulation, and is subsequently cleared via the kidney. Although we were then not able to measure SKALP/elafin in serum, this was a likely route, because SKALP/elafin is a low-molecular-weight cat-ionic protein that will easily cross basal membranes.

In the present study there were two objectives: first, we wanted to develop an assay for SKALP/elafin assessment in serum of psoriatic patients, and second we wanted to investigate if measurement of SKALP/elafin in urine and serum could be used as a marker for disease activity during therapy. In our previous study, we used a functional biochemical assay to measure SKALP/elafin in urine [13]. However, in serum this is not possible due to interfering factors. We therefore developed an enzyme-linked immunosorbent assay (ELISA) that allows measurement of SKALP/elafin both in serum and in urine. Using this ELISA we found that patients with severe psoriasis during therapy with cyclosporin A (CyA) showed a decrease of SKALP/elafin activity in serum that correlated with a decrease in clinical scores (psoriasis area and severity index (PASI) score). These findings suggest that SKALP/elafin can be used as a
marker for disease activity and can be used as a quantitative measure to monitor therapeutic effects.

MATERIALS AND METHODS

Chemicals  
TWEEN-20 was obtained from Merck, Schuchardt, Hohenbrunn bei Munich, Germany, and bovine serum albumin (BSA) from Organon Teknika, Boxtel, The Netherlands. Swine-anti-rabbit peroxidase was obtained from Dako, Copenhagen, Denmark, and o-phenylenediamine.  

2HCl from Pierce, Rockford, IL. Microtiter plates (96 wells) for ELISA were obtained from Greiner, Alphen a/d Rijn, The Netherlands. Recombinant SKALP/elafin was a kind gift from Dr. Norman Russell, ICI Pharmaceuticals, UK.

Subjects, Sample Collection and Preparation  
Fifteen healthy volunteers (5 male, 10 female; ages 2-79 years) took part in the experiments to study age or sex differences in SKALP/elafin serum levels. For determination of circadian variation blood was drawn every 4 h during 24 h in a prospective patient and a healthy control. Six male patients (ages 36-64 years, mean age 48 years) with severe chronic disfiguring psoriasis participated.

Patients had not used any systemic treatment for at least 4 weeks or local treatment for at least 1 week prior to the start of CyA therapy. In all patients, initial dosages of CyA was 3 mg/kg body weight; in two patients this dosage was increased up to 4 mg/kg body weight after 8 weeks of treatment. Before treatment, disease activity was evaluated and expressed using the PASI score. This scoring system estimates disease activity depending on erythema, infiltration, desquamation, and the area of the psoriatic skin that is affected. At the start of CyA therapy, and after 2, 4, 8, 12, and 16 weeks, samples of blood and urine were taken, and a PASI score as measure of disease activity. Samples of blood and urine were frozen and stored at −20°C until measurement of SKALP/elafin.

Anti-SKALP/Elafin Serum  
Recombinant SKALP/elafin was used for immunization procedures as described before. In short, a rabbit was immunized intracutaneously with recombinant SKALP/elafin that was crosslinked with glutaraldehyde and emulsified in Freund’s complete adjuvant. A booster with the same preparation was given after 2 weeks, and 4 weeks later serum was collected via standard methods. Control (pre-immune) serum was drawn before the immunization procedure. The specificity of the antisera was validated as described before.

ELISA  
To measure SKALP/elafin concentrations in serum and urine of psoriatic patients, we used a competitive-type ELISA. Blood samples were taken, coagulated for 60 min at room temperature, and centrifuged at 450 Xg for 10 min. Serum was acidified to a final concentration of 0.05 N HCl, boiled for 2 min, and centrifuged for 60 min (2000 Xg, 4°C). Urine was boiled for 2 min, cooled on ice for 30 min, and centrifuged for 15 min (2000 Xg, 4°C). Creatinine measurement was done before an alkaline picrate determination with kinetic endpoint detection, and carried out with reagents from Boehringer Mannheim using a Hitachi 747 analyzer. Supernatants were taken for quantification of SKALP/elafin.

The supernatants were mixed to contain 80% supernatant, 0.1 M Tris, 0.1% Tween-20, 1% BSA, and rabbit anti-SKALP/elafin antisera (1:9500 diluted), and incubated overnight. Microtiter plates (96 flat-bottom wells) were coated overnight with 50 ng/ml recombinant SKALP/elafin in phosphate-buffered saline (PBS). After washing of the plates with PBS/0.05% BSA, microtiter plates were blocked, probed with the test samples, and developed during 60 min with swine anti-rabbit peroxidase using o-phenylenediamine.2HCl as chromogenic substrate for 30 min. Human recombinant SKALP/elafin in PBS with 0.1% BSA was used as a standard:

calculation curve was made, using recombinant SKALP/elafin in the range of 0.6-80 ng/ml. The SKALP/elafin concentrations in serum or urine samples were read from this curve. All ELISA steps were performed at 4°C, except development with o-phenylenediamine 2HCl, which was done at room temperature. Data were read with a Biorad ELISA-reader, and evaluated using the Excell spread sheet program.

Statistical Analysis  
Regression analysis was performed to correlate SKALP/elafin levels in serum and urine with each other, with time of CyA treatment, and with the disease activity as expressed in the PASI score. Mean r (Pearson moment product correlation) was obtained after Fischer-z transformation.

RESULTS

PASI Score and CyA Treatment  
All patients showed good clinical improvement during 16 weeks of treatment with CyA, and apart from slight gastrointestinal discomfort no complaints were reported. Mean PASI score was 20.0 (10.6-36.0) at the start of the treatment, and decreased to 3.4 (2.0-5.4) at week 16. In two patients dosage of CyA was increased at week 8 due to lack of clinical progress. Using routine blood tests for liver and kidney functions, no side effects on these organs could be demonstrated; one patient showed hypertrichosis. Urine and blood samples of one of the patients could not be taken until 2 weeks after the start of the CyA treatment.

Sensitivity and Accuracy of the ELISA  
The ELISA we developed is of the competitive type, based on the displacement of a defined amount of recombinant SKALP/elafin by SKALP/elafin in the urine or serum samples. A calibration curve from which the sample values are read in the interval between 3-40 ng per ml is given in Fig 1. The detection limit is 3 ng/ml.

Experiments to check the recovery of recombinant SKALP/elafin added to normal human serum revealed that recovery is temperature dependent (not shown). When the ELISA was performed at 4°C a recovery of 100% was found (Fig 2). Figure 2 also shows that a pool of normal human serum has a background level of SKALP/elafin, which appeared to be about 9 ng/ml (Intercept of the Y-axis). The standard deviation of measurements in quadruplicate was within 10%.

SKALP/elafin Measurement in Urine  
SKALP/elafin levels were expressed in ng/ml urine, and corrections for creatinine concentrations were made. SKALP/elafin levels in urine varied from 3 ng/ml up to more than 120 ng/ml (Fig 3). Five of six patients showed a clear decrease in urinary SKALP/elafin levels. One patient showed extremely low values of creatinine concentration, which had an enormous impact on correction for creatinine (Fig 3c); note that his serum level of SKALP/elafin decreased during treatment (Fig 4). Decrease of SKALP/elafin levels in urine during treatment was statistically significant (Fisher-z, p < 0.05).

Urine SKALP/elafin levels decreased, but were not found below 3 ng/ml. Healthy controls do show a similar amount of SKALP/elafin in urine (data not shown), and 3-10 ng/ml may be regarded as a normal background level of SKALP/elafin in human urine.

SKALP/elafin Measurement in Serum  
Serum levels for SKALP/elafin were measured in 15 healthy volunteers. No effect of age on SKALP/elafin levels was found (not shown). A small difference in SKALP/elafin serum levels (ng/ml) was found between male (14.6 ± 2.6) and female (8.5 ± 6.1). No circadian rhythm was observed (not shown). SKALP/elafin levels in psoriatic patients varied from 11 ng/ml up to more than 300 ng/ml (Fig 4). These levels significantly decreased during treatment with CyA (Fisher-z, p < 0.05) and correlated well with clinical improvement as expressed in the PASI score (Fisher-z, p < 0.01). This decrease in SKALP/elafin level was found in the serum of all six patients. SKALP/elafin levels in serum were about sixfold higher than in urine, and levels in serum were positively correlated with levels in urine (Fisher-z, p < 0.01).

Figure 1. Calibration curve of recombinant SKALP/elafin. A sigmoid relationship between the ELISA signal and the SKALP/elafin concentration of the samples is found. The useful range for measurement of SKALP/elafin concentrations is between 3 and 40 ng/ml.
DISCUSSION

Increased amounts of SKALP/elafin have been demonstrated in urine of patients with psoriasis [13] and several other inflammatory skin diseases [14]. Because SKALP/elafin is barely detectable in normal skin and is strongly expressed in inflamed skin, we hypothesized that the increase in urinary SKALP/elafin in psoriatics is derived from local production in skin. This would be in line with the fact that SKALP/elafin is a secreted molecule [16] and the notion that epidermis acts as a secretory organ as recently described [17,18]. However, no definitive proof could be given for the epidermal origin of urinary SKALP/elafin in psoriatic patients, because SKALP/elafin could not be demonstrated in the intermediate compartments between epidermis and urine (i.e., dermis and blood). In our initial study we used a functional assay to measure SKALP/elafin, that could not be applied to serum, due to interference by the overwhelming amount of other plasma-derived proteinase inhibitors.

Intraepidermal accumulation of PMN is an early event in the pathogenesis of the psoriatic lesion [19], and PMN-derived proteinases were shown to degrade the dermo-epidermal junction in vitro [20]. This suggests that elastase may be involved in PMN migration and PMN-dependent tissue damage and may promote the inflammatory response. Several antipsoriatic therapies are known to interfere with this PMN migration, suggesting that they are involved in the downregulation of inflammation [21]. In addition to proteinases from inflammatory cells, keratinocyte-derived proteinases such as plasminogen activator may act as a pro-inflammatory mediator in psoriatic skin [22]. Synthesis of proteinase inhibitors that counteract proteinases by forming inactive complexes is one of the in vivo mechanisms to protect tissue against unwanted proteolysis [23]. These proteinase inhibitors may be present in plasma, or can be produced locally at the site of action [8,24,25]. Complexes between elastase and elastase inhibitors in serum, sputum, bronchoalveolar lavage, and urine have been described, the inhibitors being α1-PI [26–28], antileukoproteinase [29], or SKALP/elafin [13]. Levels of these complexes may be related to disease activity, and measurement may provide a tool for monitoring therapeutic result. Increased levels of complexes between elastase and inhibitors, but also of free elastase or free inhibitor, have been reported in lung cancer [30], neonatal systemic infection [31,32], adult respiratory distress syndrome [27,32], cervical cancer [33,34], psoriasis, eczema, and other cutaneous disor-

Figure 2. Mean values ± 2 SD of measurements in quadruplicate of three concentrations of recombinant SKALP/elafin in normal human serum. SD is 10%. The intercept with the Y-axis indicates that in humans a background serum level of SKALP/elafin appears to be present. This background level is about 9 ng/ml.

Figure 3. SKALP/elafin levels in urine of six psoriatic patients (a–j) during treatment with CyA. SKALP/elafin levels (ng/ml) are shown before (open squares) and after correction for creatinine concentration (solid squares). Decrease of SKALP/elafin levels in urine is statistically significant (Fisher z, p < 0.05).
Figure 4. SKALP/elafin levels in serum of six psoriatic patients (a-f) during treatment with CyA. Amount of SKALP/elafin in serum is given in ng/ml (solid squares). Decrease of SKALP/elafin levels in serum is statistically significant (Fisher z, p < 0.05). PASI scores at time of sampling are given (open squares).

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