

Appendix S1

MATERIALS AND METHODS

Patients

Twenty-eight patients with AD, 18 females and 10 males, mean age 29.5 years (age range 19–48 years) were recruited as patients with ongoing AD according to Williams et al. (20) after being referred by their family doctors to our clinical department. The patients did not receive systemic therapy (including phototherapy and antihistamines) within one month prior to initiation of the study. They did not have a history of being treated for mental disorder/depression.

Ethical permission was obtained from the local ethics board.

SCORAD

The extent of the disease was determined using SCORing of Atopic Dermatitis (SCORAD) (21). Both objective and subjective SCORAD were determined.

Pruritus intensity

Pruritus intensity was determined using a visual analogue scale (VAS), linear 0–10.

Psychodemographic data

The patients' personality traits were evaluated using the Swedish Universities Scales of Personality (SSP) (22), a 91-item questionnaire, completed by the patients and analysed regarding somatic trait anxiety, psychic trait anxiety and stress susceptibility. Instructions on how to complete the forms were given by the physician (KN). Absolute values were calculated. For depression score, Montgomery–Åsberg Depression Rating Scale-Self assessment (MADRS-S) (23) was used.

Processing of biopsy specimens

Biopsies of 3 mm thickness were taken from lesional (L) skin (with dryness, papules, often lichenified) of the elbow, and from non-lesional (NL) skin from the lower back region. No topical steroids had been used on any of these areas for at least 14 days. Lana's fix (phosphate buffered 4% formaldehyde containing 0.2% picric acid) was used for fixation of the biopsies for 2 h at 4°C. After fixation they were rinsed in 0.1 M Sörensen's phosphate buffered saline (PBS) supplemented with 10% sucrose for at least 24 h and then rapidly frozen and stored at –70°C until further processing. While still frozen the biopsy tissue was placed in a tissue holder and embedded in Tissue Tek OCT Compound (Sakura Finetek, Zoeterwoude, the Netherlands). A Microm HM500 cryostat (Heidelberg, Germany) was used for cutting the biopsies into 14 µm thick sections and these were mounted onto Super Frost slides for immunohistochemical staining using a biotinylated-streptavidin procedure.

Immunohistochemical analysis

Sections prepared as described above were incubated with 10% normal goat serum (S 100, Vector, Burlingame, CA, USA) for 40 min and thereafter with rabbit polyclonal antibodies against 5-HT (20080, dilution 1:10,000; Diasorin, Stillwater, MN, USA) or 5-HT1AR (SIA-170; 1:1,000) overnight at 4°C. The 5-HT1AR antibody is directed against amino acids 170–186 in the second extracellular loop (24). Alternatively, the sections were incubated with 10% normal horse serum (S-2000, Vector) followed by

mouse monoclonal antibodies toward SERT (ST51-5; 1:10,000; MabTechnologies, Stone Mountain, GA, USA) directed against amino acid residues 51–66 coupled to keyhole limpet haemocyanin (KLH) through an additional N-terminal cysteine residue, or 5-HT2AR (sc-166775; 1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Thereafter, incubation with biotin-labelled anti-rabbit (BA-1000) or anti-mouse (BA-2000) IgG (both diluted 1:200; Vector) as the secondary antibody was performed for 40 min at room temperature followed by treatment with the fluorochrome Cy2-labelled streptavidin (PA42001, 1:2,000; Amersham Pharmacia Biotech, Uppsala, Sweden), also for 40 min at room temperature. All antibody solutions were diluted in PBS containing 1% bovine serum albumin (BSA) (A9418, Sigma-Aldrich, Stockholm, Sweden) prior to use.

Control staining with non-specific mouse IgG of the same isotype (X0931, Dako, Glostrup, Denmark) and in the same dilution as the monoclonal antibody against SERT or 5-HT2AR, was negative. In the case of polyclonal antibody toward 5-HT, pre-adsorption with this compound (85030; Sigma-Aldrich) at a concentration of 10⁻⁶ mol/l eliminated the staining. Moreover, when the primary antibodies were omitted a substantial reduction or no staining was recorded.

Microscopy

The sections were evaluated by 2 observers who were blinded as to from which patient the sections had been obtained. The observer's scores were similar.

Of the general histopathological changes, hyperkeratosis, acanthosis and degree of infiltration by inflammatory cells in the dermis were graded semiquantitatively, 0–3 (0=normal appearance, 1=mild, 2=moderate and 3=severe). The degree of 5-HT immunoreactivity was determined semiquantitatively, 0–3 (0=no signal, 1=slight, 2=moderate and 3=strong) in the epidermis and inflammatory infiltrates, while the absolute number of 5-HT-positive platelets was determined. An average of 4 sections was calculated for each biopsy.

For 5-HT1AR the fraction of positive staining of the total thickness of epidermis was evaluated, 0=0%, 1≤25%, 2≤50% and 3≤75%. The absolute number of positive 5-HT1AR inflammatory mononuclear cells in the papillary dermis was determined.

For 5-HT2AR the epidermal fraction expressing this receptor of the total thickness of epidermis was, similarly as the 5-HT1A epidermal fraction, graded 0–3. Basement membrane staining intensity was graded 0 for no staining, and 1–3 for increased staining intensity, 1=slight, 2=moderate and 3=strong staining. The number of vessels expressing 5-HT2AR in papillary dermis was graded 0–3 (0<40, 1=40–59, 2=60–79 and 3≥80 vessels per section).

The absolute number of SERT-positive mononuclear cells was counted in the epidermis and papillary dermis, respectively. In addition, the SERT signal intensity of the basal epidermal layer was assessed 0–3, as 0=minimal, 1=slight, 2=moderate and 3=strong staining.

Statistical analysis

Processing of the absolute numerical data was performed to determine if there was any difference between L and NL skin. The Student's *t*-test or a non-parametric test was used to analyse these data. For the semi-quantitative data the χ^2 test and/or Fisher's exact test were used in non-dependent samples, and the Student's *t*-test or non-parametric test was used for dependent samples. Correlations between the different parameters were determined using Pearson's or Spearman's test, depending on whether the values were normally distributed or not. Differences were considered to be statistically significant at *p*<0.05.