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INVESTIGATIVE REPORT

The Helix-Loop-Helix Transcription Factor Id1 is Highly Expressed in Psoriatic Involved Skin

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The helix-loop-helix transcription factor Id1 (inhibitor of differentiation/inhibitor of DNA binding) functions as an inhibitor of differentiation. We have examined Id1 gene expression in cultured keratinocytes in punch biopsies from psoriatic involved and uninvolved skin, and in skin specimens from normal individuals. Id1 mRNA expression was measured with an RNase protection assay and with Northern blot. Id1 immunoreactivity was determined in skin biopsies by immunofluorescence using a polyclonal antibody directed against the Id1 protein. In cultured keratinocytes, the expression of Id1 mRNA was strongest in small cells with high proliferative potential, whereas in large cells, which are terminally differentiated, the expression was low. Expression of the Id1 mRNA in psoriatic involved skin ($n=9$) was significantly elevated compared to uninvolved skin from the same patient ($n=5$) and to skin from normal controls ($n=9$). Id1 immunoreactivity was intranuclear throughout all the layers in psoriatic involved epidermis, except in the stratum corneum, while no immunoreactivity was detected in uninvolved epidermis. In normal controls, cytoplasmatic Id1 immunoreactivity was detected in the basal layer in epidermis obtained from newborns, while no immunoreactivity was detected in epidermis obtained from the adults in the control group. We conclude that Id1 is expressed in cells with high proliferative potential, and is downregulated in cells that undergo terminal differentiation. Along with the overexpression of the Id1 gene in psoriatic involved skin, these observations suggest that Id1 is involved in the process of differentiation of keratinocytes seen in normal skin and that the Id1 pathway is activated in psoriasis. ***Key words: Id1: helix-loop-helix protein; psoriasis.***

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More knowledge of the genes that regulate the process of keratinocyte differentiation is of potential interest. It has been shown that keratinocytes of the basal epidermis express basonuclin, a regulatory zincfinger protein specific to keratinocytes that is linked to maintenance of the proliferative capacity of the keratinocytes and disappears from the cell prior to terminal differentiation (1). Li et al. (2) have cloned a basic helix-loop-helix (HLH) protein called Dermo-1 which is mainly expressed in the dermis. It has been suggested that this functions as a regulator of gene expression in a subset of mesenchymal cell lineages.

Transcription factors belonging to the family of HLH proteins are nuclear proteins that regulate gene expression accompanying the process of cell proliferation and differentiation (3). The HLH proteins are divided into 3 subclasses: class A proteins are widely expressed and dimerize preferentially with class B proteins, which are cell-type-specific. Examples of class A HLH proteins are E12 and E47 (4), while examples of class B proteins are the myogenic regulatory factors MyoD, Myogenin and Myf5 (5). Class A and class B HLH proteins comprise a basic region that mediates the binding of the heterodimers to an E-box motif present in the enhancers or promoters of the tissue-specific genes.

Other members of the HLH family are the Id proteins (inhibitors of differentiation/inhibitor of DNA binding). The Id proteins lack the basic region and act as negative regulators of differentiation by competitive inhibition of heterodimer formation between the basic tissue-specific HLH proteins and the E-proteins. The Id protein pool is thought to be localized in the cytoplasm and when activated Id binds to a basic HLH protein and is subsequently translocated to the cell nucleus (6). The Id1 protein was first isolated in mouse, and it has been sequenced and functionally characterized (7). Today there are 4 known human Id proteins, Id1–Id4 (8–12). The objectives of this study were to examine whether normal human keratinocytes express the HLH protein Id1, and to investigate if this negative regulator of differentiation is involved in the differentiation abnormalities seen in psoriasis.

MATERIALS AND METHODS

Acquisition of tissue

Patients with mild or moderate psoriasis with no ongoing treatment for their psoriasis in the previous 3 weeks and with

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new and active psoriatic plaques were selected from the outpatient clinic of the Department of Dermatology, Sahlgrenska University Hospital, Göteborg and from the Swedish Psoriasis Genetic Project (13). The control group consisted of personnel working in our laboratory or patients undergoing plastic surgery (breast reduction or abdominal plasty). Skin tissue from patients and controls was taken, in the spring, from comparable sites on untanned skin. Punch biopsies (4 mm) were used with the subjects under local anaesthesia (1% lidocaine without adrenaline). In 6 psoriatic patients, punch biopsies were taken from involved and uninvolved skin, and in 3 from involved skin only. One of the skin biopsies obtained from psoriatic uninvolved skin was excluded because of RNA degradation. Normal leftover skin from newborns was taken during circumcision surgery.

The dermis was macroscopically excised when the tissue was used for RNA and protein preparation, and the samples were immediately frozen in liquid nitrogen. Patients and control subjects gave their informed consent to the procedure, and the study was approved by the Ethics Committee of the Medical Faculty, Göteborg University.

Cell culture

Human epidermal keratinocytes derived from foreskin of a normal newborn (strain YF29) were grown with supporting 3T3-J2 cells (14) using additives to the culture medium (15, 16), including 10% fetal calf serum (Hyclone, Logan, UT). Megacolonies of keratinocytes were grown on 150 mm dishes by inoculating 10–20 cells/dish and cultivating for 16–18 days (1). Well-isolated colonies were then chosen for the experiments.

Centrifugal elutriation

Keratinocytes were grown to confluence in 150 mm dishes, trypsinized and collected by centrifugation. Centrifugal elutriation was performed as described previously and a total of 1.5×10^8 cells were used (1). Seven fractions of cell suspensions containing $1-2 \times 10^7$ cells were collected. A drop of each cell suspension was photographed in a hemacytometer for size measurement and cell counts. The remaining cells were lysed for RNA preparation.

Northern analysis

Total RNA was isolated from cells using the RNeasy Total RNA kit (Qiagen Inc., Santa Clarita, CA, USA). Total RNA (10 μg) from each cell fraction was loaded in each lane, separated on a 1% formaldehyde-agarose gel and transferred to nylon membranes (Amersham, Bucks, UK). Blots were prehybridized and hybridized at 68°C in Quickhyb hybridization solution (Stratagene Corp., La Jolla, CA, USA). The probes were labelled with ^{32}P dCTP using a multiprime labelling system kit (Amersham, Bucks, UK). The RNA was first probed with a 263 bp fragment corresponding to Id1 cDNA (17) and then dehybridized and reprobed sequentially with cDNAs encoding the human basonuclin and involucrin (1, 18). 18S and 28S rRNAs probed with specific oligonucleotides were used as controls for loading variation and RNA integrity.

Ribonuclease protection assay (RPA)

A riboprobe of 298 bp for Id1 was generated as described previously (19). Id1 gene expression was analysed with an RPA, using the Ribonuclease Protection Assay Kit (Ambion Inc., Austin, TX, USA). The samples were hybridized with the probe at 45°C. RNase digestion was carried out at 37°C for 30 min. Samples consisted of 5–7 μg total RNA isolated from punch biopsies taken from normal skin and from psoriatic involved and uninvolved skin. Negative controls consisted of yeast tRNA. ^{33}P-labelled HaeIII DNA (Promega, Madison, WI, USA) was used as a molecular size marker. Protected fragments were separated on a 6% polyacrylamide gel (Novex San Diego, CA, USA) and the gels were exposed to Phosphor Imager Screens. The screens were developed on a phosphorimager (Phosphorimager, Molecular Dynamics).

Immunofluorescence

Cryostat sections were subjected to immunostaining according to the following schedule: Fixation in cold absolute acetone/methanol (1:1) for 15 min at 20°C; quenching of endogenous peroxidase with 3% H_2O_2 in phosphatase buffered saline (PBS) for 10 min; washing 3 times in PBS; blocking with Avidin D blocking solution for 15 min and with Biotin blocking solution for 15 min at 20°C (Avidin Blocking Kit, Vector Laboratories, CA, USA); a final blocking with 1 × blocking solution from the DIG wash and block buffer set (Roche Diagnostics, Mannheim, Germany) for 30 min at 20°C; incubation overnight at 4°C with the Id1 rabbit polyclonal antibody (C-20 Rabbit polyclonal 200 μg/ml Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:1500; washing 3 times in 0.05% Tween 20 in PBS; incubation for 1 h at 20°C with Sheep anti-Rabbit POD diluted 1:100 (Santa Cruz); washing 3 times in 0.05% Tween 20 in PBS; incubation for 10 min at 20°C with FT working solution (TSA-direct Cy3 kit, Dupont, NEL); washing 3 times in 0.05% Tween 20 in PBS; mounting in DAPI-containing mountain medium (Vector Labs).

Negative controls were replacement of primary antibody by unrelated rabbit serum. In blocking experiments performed in addition, the recombinant Id1 peptide was added to a final concentration of 10 times excess, after which immuno-incubation was performed. This resulted in abolition of the immunostaining (data not shown).

Western blot

Western blot was performed on additional punch biopsies obtained from adult normal and psoriatic involved and uninvolved skin. Total protein extracts were prepared by sonicating skin tissue in 1 mM dithiothreitol (DTT). The protein concentration was determined using the protein assay kit (Bio-Rad, Hercules, CA, USA). Equal volumes protein/DTT and 2 × Novex sample buffer (Novex, San Diego, CA, USA) were mixed and 10 μg of proteins was loaded in each lane of a 14% Tris-glycine gel. The proteins were size-fractionated on the gel and then electrophoretically transferred onto a nitrocellulose membrane (Novex). The membrane was blocked overnight in 10% dry milk and 0.1% Tween 20 in Tris-buffered saline (TBS) and probed with anti-Id1 (Santa Cruz) diluted 1:250. After washing in 0.5% Tween 20 in TBS (TBS-T) the membrane was incubated with anti-rabbit Ig, horseradish peroxidase linked $F(ab')_2$ fragment (Amersham, Bucks, UK), diluted 1:10 000 . The membrane was washed in TBS-T and TBS, and finally the immunoreactive proteins were visualized with an ECL Western blotting detection system (Amersham).

Statistical procedures

The Mann-Whitney U test was used when comparing psoriatic skin with skin from normal controls. Wilcoxon's signed-ranks test was used when comparing psoriatic involved skin with psoriatic uninvolved skin.

RESULTS

Expression of the Id1 mRNA in cultured keratinocytes separated according to size

To study changes in abundance of Id1 mRNA in cultured keratinocytes during their enlargement and terminal differentiation, confluent cultures of keratinocytes were trypsinized and the cells were separated according to size by centrifugal elutriation. Northern analysis of total RNA prepared from elutriation fractions was performed (Fig. 1). The first 3 fractions, which contained small cells, expressed Id1 and basonuclin mRNAs, whereas the last 4 fractions, which contained keratinocytes with a mean cell size >22 μm, expressed involucrin mRNAs. In the last 4 fractions the Id1 expression was diminished. The integrity of the RNAs and the loading variations were verified by probing the blot with oligonucleotides corresponding to 18S and 28S ribosomal RNAs.

Id1 mRNA expression in normal skin and psoriatic involved and uninvolved skin

RPA was used to measure Id1 mRNA expression in normal skin and in psoriatic involved and uninvolved skin. A protected fragment with the expected length was obtained when total RNA was hybridized with the Id1 probe. There was no significant difference in the expression levels of Id1 between uninvolved skin and skin from normal controls. However, we found statistically significant elevated expression levels of Id1 in all of the psoriatic involved skin ($n=9$) compared to skin from the normal control group ($n=9$). Furthermore, there was a significant elevation of Id1 expression in a subgroup of 5 of the psoriatic patients when Id1 expression in involved skin was compared to uninvolved skin from the same patient (Fig. 2a). To ensure that the difference of expression level observed was not due to different amounts of RNA

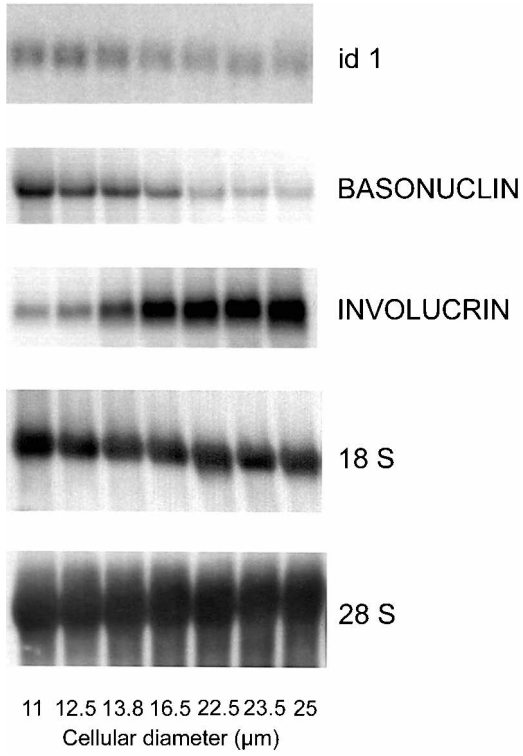

Fig. 1. Small cells contain Id1 and basonuclin mRNAs, while large cells contain involucrin. Confluent cultures of keratinocytes were trypsinized and separated according to size by centrifugal elutriation. Northern analysis of total RNA prepared from each elutriation fraction was performed. The integrity of the RNAs and the loading variations were verified by probing the blot with oligonucleotides corresponding to 18S and 28S ribosomal RNAs.

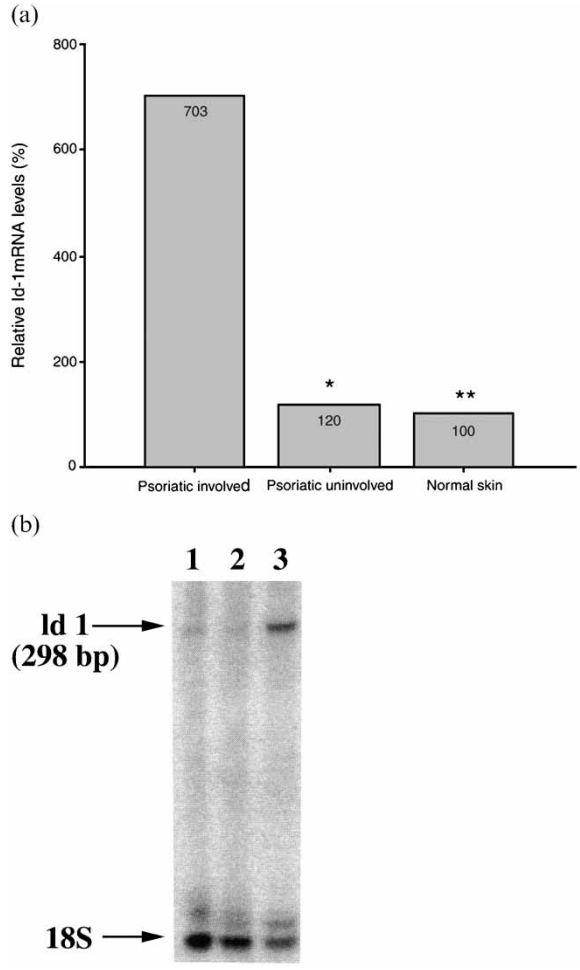

Fig. 2. The Id1 mRNA is overexpressed in psoriatic involved skin compared to normal skin. (a) The samples were analysed by ribonuclease protection assay and quantified by densitrometric analysis on a phosphorimager. The relative RNA levels of Id1 in psoriatic involved skin were expressed as a percentage of the expression in normal controls (100%). Expression levels of Id1 in psoriatic involved skin ($n=9$, mean 703, range 93–2440) compared to normal skin ($n=9$, mean 100, range 19–246) were significantly higher ($^{**}P<0.01$). Expression levels of Id1, in a paired comparison, between psoriatic involved skin and psoriatic uninvolved skin ($n=5$, mean 120, range 46–310) were significantly higher ($^{*}P<0.05$). (b) Expression levels of Id1 mRNA in psoriatic skin compared to normal controls demonstrated by ribonuclease protection assay; Lane 1: normal skin; Lane 2: psoriatic uninvolved skin; Lane 3: psoriatic involved skin. Similar expression levels of Id1 in psoriatic involved and uninvolved skin compared to normal skin were seen in all the patients.

loaded on the gel, 18 S was used as an internal standard in one additional RPA (Fig. 2b). In 4 of the normal controls we were unable to detect Id1 expression by RPA, but mRNA expression could be demonstrated by RT-PCR (data not shown).

Id1 protein immunoreactivity in normal, psoriatic involved and uninvolved skin

Immunofluorescence was performed in order to study the Id1 protein distribution. In psoriatic involved skin, nuclear Id1 immunoreactivity was present in all the epidermal layers except the stratum corneum (Fig. 3b, c). In normal skin, no immunoreactivity was detected in epidermis obtained from adults in the control group (Fig. 3a). while basal cytoplasmatic staining was observed in epidermis obtained from normal newborns (Fig. 3e). No immunoreactivity was found in uninvolved skin (data not shown).

Furthermore, a Western blot was performed. The antibody detected a band of the expected size slightly

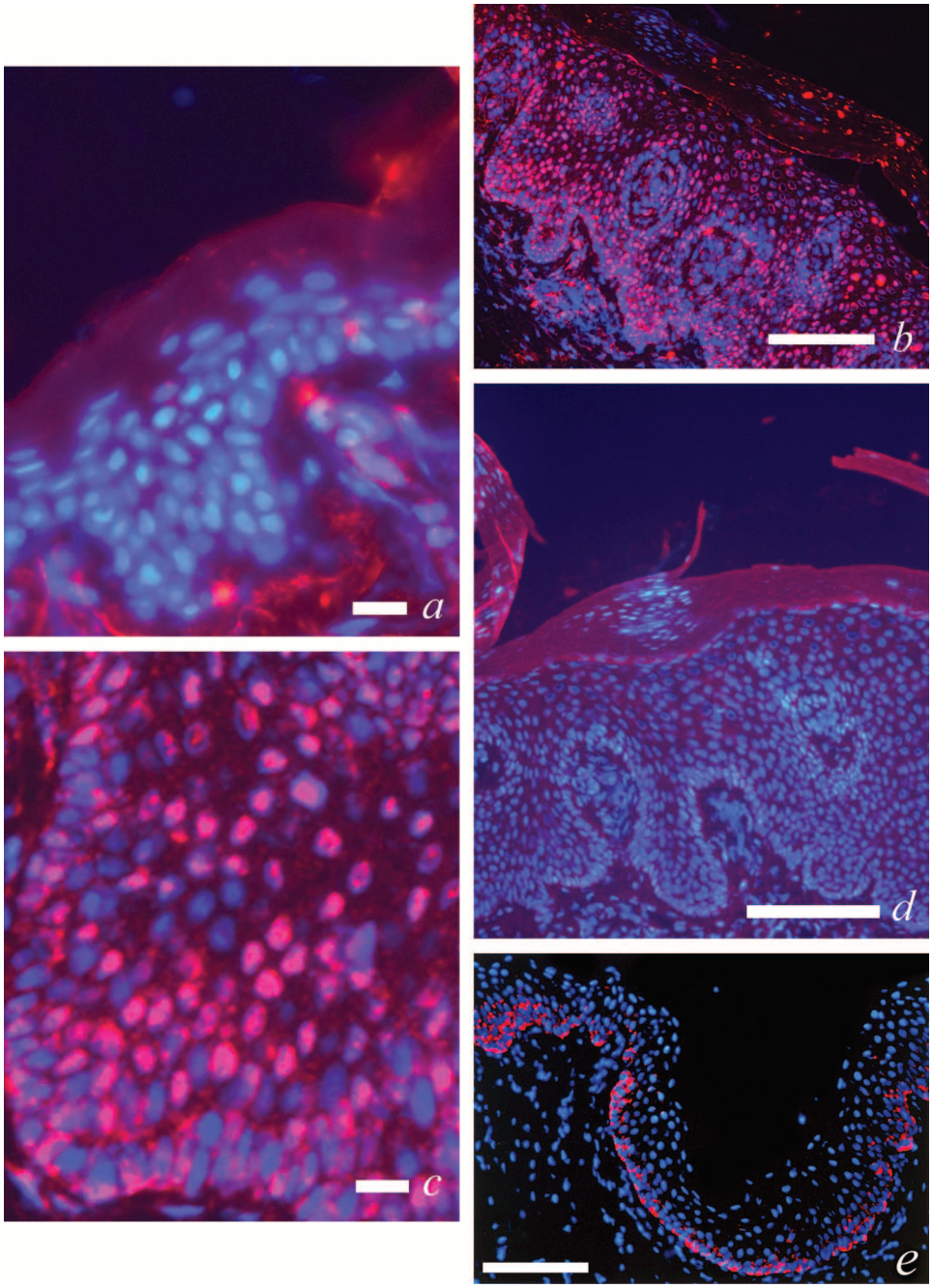

Fig. 3. Intranuclear immunostaining for Id1 in all layers of psoriatic involved epidermis, except for the stratum corneum. Cryosections from normal and involved psoriatic skin were immunostained for Id1. (a) Normal skin, obtained from the control group, immunostained for Id1. (b, c) Involved psoriatic skin immunostained for Id1. (d) Involved psoriatic skin incubated with non-immune rabbit sera. (e) Normal skin obtained from newborn, immunostained for Id1. Scale bar 10 μm (a, c); 100 μm (b, d, e).

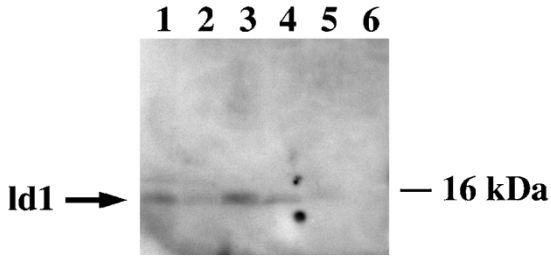

Fig. 4. Id1 protein expression demonstrated by Western blot. Expression of Id1 protein in psoriatic skin compared to normal skin, demonstrated by Western blot. Lanes 1 and 3: psoriatic involved skin; Lanes 2 and 4 psoriatic uninvolved skin; Lanes 5 and 6: normal adult skin.

less than 16 kDa. The Id1 protein was more strongly expressed in psoriatic involved skin compared to uninvolved and normal skin. No band was detected in one of the normal subjects (Fig. 4)

DISCUSSION

In several cell types, the differentiation programme has been shown to be dependent on the activity of basic HLH proteins (20, 21). In addition, the Id genes have been shown to be strongly expressed in cell lines derived from a variety of different tumours, e.g. chondrosarcoma, neuroblastoma, neuroepithelioma and lung carcinoma (10, 19, 22), and overexpression of Id1 in muscle precursor cells, myeloid precursor cell lines or mammary epithelial cells inhibits their differentiation (7, 23, 24). In addition, Alani et al. (25) reported that ectopic overexpression of Id1 leads to activation of telomerase activity and immortalization of primary human keratinocytes. However, Nickoloff et al. (26) reported that Id1 delays senescence but does not immortalize keratinocytes.

We have investigated the expression of Id1 in cultured keratinocytes and in human normal and psoriatic skin. An efficient way of studying the correlation of Id1 expression with keratinocyte differentiation is to use a single time-point culture, where confluent keratinocytes are separated according to size by centrifugal elutriation. This allows specific analyses of keratinocytes at different stages of differentiation. When this was performed and Id1 mRNA was analysed by Northern blot, we found that the expression of Id1 mRNA was strongest in small cells with a mean diameter of 11–13.8 μm. This population also contained basonuclin mRNA, a regulatory zincfinger protein specific to keratinocytes, linked to the maintenance of proliferation (1). Cells of this size are known to include nearly all the colony-forming cells of the culture (1, 27, 28). In cells >22.5 μm in diameter, the abundance of Id1 mRNA was diminished, as was that of basonuclin mRNA. These cells accumulated involucrin mRNA, a characteristic of terminal cell differentiation (29). Cells of this size range do not synthesize DNA (30) and are unable to form colonies (1, 27, 28). These data suggest that in epidermal cultures the Id1 gene is mainly expressed in cells with high proliferative potential, whereas Id1 expression is downregulated in cells undergoing terminal differentiation.

To investigate whether these observations had any association with a human cutaneous proliferative disease, the expression of Id1 mRNA in punch biopsies from psoriatic patients was measured and compared with normal controls. We found an overexpression of Id1 mRNA in involved skin from psoriatic patients compared to uninvolved skin and skin from normal controls. There was no significant difference in Id1 mRNA expression in uninvolved skin from psoriatic patients compared to skin from normal controls.

When Id1 protein distribution was investigated by immunofluorescence, there was basal cytoplasmatic Id1 immunostaining in epidermis from normal newborn foreskin. However, there was no immunostaining in skin sections obtained from the control group. This concurs with the low mRNA expression in the control group, where mRNA was detected by RT-PCR but not by RPA in 4 subjects.

Other groups have found varying results regarding Id1 immunoreactivity in skin. Langlands et al. (31) reported cytoplasmatic Id1 protein localization in the basal layers of normal epidermis and some nuclear staining in the spinous and granular layers, whereas Schaefer et al. (32) did not detect any immunostaining in normal or psoriatic involved skin in 3 patients. The varying results may be caused by low and varying Id1 expression in normal skin as part of the local homeostasis control of the epidermis, and may reflect a difference in proliferation potentials depending on for example the age of the patient or the activity of the plaque. The Western blot performed in this study on additional punch biopsies obtained from adult normal and psoriatic involved and uninvolved skin supports the fact that expression differs, since in these samples there was a vague band about 16 kDa in uninvolved skin and in one of two of the normal controls compared to the stronger band in the 2 samples obtained from involved psoriatic skin. A different performance of the Id1 antibody in Western blot and immunostaining analysis can be another explanation.

It is not clear whether the patients in the study by Schaefer et al. (32) were treated or not, or if the psoriatic plaques were newly developed. We found intranuclear immunostaining in all epidermal layers, except the stratum corneum, in skin sections obtained from newly developed active psoriatic plaques. The immunostaining varied in the basal layer in different parts of the sections. It may not be expected that the Id1 expression strictly must be linked to the grade of differentiation of the keratinocytes in psoriasis, since the differentiation pathway in psoriasis may be altered

or truncated, which does not manifest itself as terminal differentiated keratinocytes. The Id1 expression may be upregulated in some of the keratinocytes when entering or during some stages of an alternative differentiation programme. Furthermore, Id1 has been shown to play a role in cell cycle control, and the different ways it affects the balance between growth and differentiation are not fully understood. It has been reported that Id3 co-transfected with its E-protein target results in translocation of the Id3 protein to the nucleus (6). The intranuclear localization of the Id1 immunostaining observed in psoriatic epidermis may indicate that a HLH partner for Id1 exists in keratinocytes and, taken together with the overexpression of Id1 mRNA in psoriatic involved skin, may suggest that Id1 is functionally active in psoriasis.

We conclude that human skin and keratinocytes express the Id1 gene and that Id1 is mainly expressed in keratinocytes with high proliferative potential and is downregulated during differentiation. Our results indicate that the Id1 protein may be involved in a regulatory pathway in the epidermis in vivo and that this pathway may be activated in psoriasis. Further studies are indicated to investigate the regulation of Id1 in epidermal keratinocytes, e.g. is the therapeutic effect of vitamin D in psoriasis linked to suppression of Id1? It would also be of interest to define target genes and binding partners for Id1 in keratinocytes.

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