A Characterization of the expression of 14-3-3 isoforms in psoriasis, basal cell carcinoma, atopic dermatitis and contact dermatitis

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Abstract

14-3-3 is a highly conserved protein involved in a number of cellular processes including cell signalling, cell cycle regulation and gene transcription. Seven isoforms of the protein have been identified; β, γ, ε, ζ, η, τ, α and τ. The expression profile of the various isoforms in skin diseases is unknown. To investigate the expression of the seven 14-3-3 isoforms in involved and uninvolved skin from psoriasis, basal cell carcinoma (BCC), atopic dermatitis and nickel induced allergic contact dermatitis. Punch biopsies from involved and uninvolved skin were analyzed with quantitative reverse transcription-polymerase chain reaction to determine the mRNA expression of the 14-3-3 isoforms. The protein level of 14-3-3 isoforms was measured by Western blot technique in keratome biopsies from patients with psoriasis. Evaluation of dermal and epidermal protein expression was performed by immunofluorescence staining. Increased 14-3-3 protein levels were detected in involved skin from patients with psoriasis, contact dermatitis and BCC. 14-3-3 mRNA expression was increased in psoriasis and contact dermatitis, but not in BCC. In atopic dermatitis no significant difference between involved and uninvolved skin was found. The expression of the 14-3-3 isoforms was also studied at the protein level in psoriasis. Only 14-3-3 protein expression was significantly increased in involved psoriatic skin compared with uninvolved skin. Immunofluorescence staining with 14-3-3 isoforms showed localization of both isoforms to the cytoplasm of the keratinocytes in the various skin sections. These results demonstrate a disease specific expression profile of the 14-3-3 protein.

Introduction

14-3-3 is a highly conserved protein family comprising seven different isoforms with molecular weights of 29-33 kDa. The 14-3-3 protein was first discovered in 1967 by Moore and Perez as an abundant brain protein and it has been found in all eukaryote organisms investigated so far. 14-3-3 is found in all tissues and interacts with more than 200 proteins. It is involved in a number of cellular processes including cell signalling, regulation of the cell cycle, intracellular trafficking, regulation of the cytoskeletal structure and control of gene transcription. In general the cellular effects of 14-3-3 can be classified into three categories: i) Directing cell conformational changes; ii) binding to target proteins and modulation of their activity and/or interactions with other cellular components; iii) acting as a phosphorylation-dependent scaffold protein that recruits other proteins. Seven different isoforms of 14-3-3 have been identified and are denominated α, γ, ε, τ, κ, β and δ. Furthermore, α and δ isoforms have been identified as phosphorylated forms of β and γ respectively. The functions of the different isoforms are not yet clarified. 14-3-3 is the best characterized isoform and is in particular expressed in epithelial cells. The expression of 14-3-3 increases in the cells after DNA damage and together with p53 it plays an important role for the G2/M checkpoint in the cell cycle. The gene can be inactivated by CpG methylation (epigenetic silencing) of the promoter region, which is seen in different types of carcinomas, including basal cell carcinoma. The 14-3-3 isoform is important in embryogenesis and both knockout mice and Xenopus with inactivated 14-3-3 do not survive embryonic development. 14-3-3; downregulates the level of p53 and is upregulated in more than 40% of advanced breast cancers. 14-3-3 and 14-3-β seem to have oncogenic potential when overexpressed. Overexpression of 14-3-3β in NIH 3T3 cells stimulate cell growth and when injected in nude mice, it augments tumor formation, while overexpression of 14-3-3γ in H322 lung cancer cells results in abnormal DNA replication and polyploidization. The expression of 14-3-3γ is upregulated in aged skin compared with young skin, and the protein is induced and accumulated after ultraviolet radiation. The expression is higher in sun-exposed skin compared with sun-protected skin from the same patient. Taken together, these findings demonstrate distinct properties of the various 14-3-3 isoforms.

All seven isoforms have been identified in human keratinocytes in vitro, whereas all isoforms except 14-3-3 were found in fibroblasts. Western blot analysis of intact human epidermis and immunohistochemical staining of human skin sections detected six isoforms but not the 14-3-3γ. Although the 14-3-3 protein is expressed in human skin the role of 14-3-3 proteins in inflammatory skin diseases has only been sparsely investigated. Using immunohistochemistry and polymerase chain reaction (PCR) technique Lodigyn et al. found similar 14-3-3 expression level in both involved psoriatic skin and normal skin, whereas 14-3-3 expression was reduced or absent in basal cell carcinoma.

The role of 14-3-3 in different common skin diseases is therefore unclear. The aim of this study was to characterize the mRNA profile of the seven 14-3-3 isoforms in four common skin diseases; i) psoriasis, a benign, Th-1 and Th-17 driven chronic inflammatory, hyperproliferating skin disease; ii) atopic dermatitis, a Th-2 driven chronic inflammatory skin disease; iii) acute, allergic nickel induced contact dermatitis; iii) basal cell carcinoma (BCC), a malignant hyperproliferating skin disorder. The protein profile of the seven 14-3-3 isoforms was only characterized in psoriatic skin because this was the only disease where sufficient tissue material could be collected allowing protein determination.
Materials and Methods

Biopsies

BCC biopsies were taken from patients with histologically verified superficial or nodular BCC. The biopsies were taken from the center of the carcinoma. Biopsies from uninvolved skin were taken from normal skin from the same body region. From patients with atopic dermatitis punch biopsies from involved and uninvolved skin were taken from the same body region. The patients did not receive any systemic therapy for four weeks while on ice the keratinocytes were washed and the supernatant constituted the cell lysate.

Equal amounts of protein (determined by Bradford) were separated by SDS-PAGE and blotted onto nitrocellulose membranes. The membranes were incubated with the following primary antibodies 14-3-3-β (catalog no. #9636), 14-3-3-γ (catalog no. #9637), 14-3-3-ε (catalog no. #9635), 14-3-3-ζ (catalog no. #9639), 14-3-3-η (catalog no. #9640), 14-3-3-σ (catalog no. #9638) from Cell Signaling Technology, Beverly, MA, USA, and 14-3-3-κ (catalog no. sc-100638) from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Antibodies were detected with horseradish peroxidase-conjugated anti-rabbit (catalog no. #7074 from Cell Signaling Technology, Beverly, MA, U.S.A.) or anti-mouse (catalog no. P0447, DAKO, Glostrup, Denmark) in a standard ECL reaction (GE Healthcare, Wessling, Germany). To estimate the protein size a biotinylated protein ladder molecular weight maker was used followed by anti-biotin (catalog no. #7727 and #7075 from Cell Signaling Technology, Beverly, MA, U.S.A.) or anti-mouse (catalog no. P0447, DAKO, Glostrup, Denmark) in a standard ECL reaction (GE Healthcare, Wessling, Germany).

Immunofluorescence

The tissue sections were deparaffinized in xylene and hydrated through a descending ethanol series. For antigen unmasking, the sections were heated at 90°C for 15 min. in a Tris/EGTA buffer (pH 9.0) followed by cooling for 20 min. To block non-specific binding sites the sections were incubated with Image IT FX signal enhancer (Molecular Probes, Invitrogen, Carlsbad, CA, U.S.A.) for 30 min. The sections were immunostained with 14-3-3-κ antibody (Cell Signaling Technology, Beverly, MA, USA no 9638) and 14-3-3-ζ antibody (Santa Cruz Biotechnology, no SC-100638) overnight at 4°C. The next day the sections were incubated with secondary antibody (Alexa Flour 488 from Molecular Probes, Invitrogen) for 1 hour. Nuclear staining was performed by mounting samples in Prolong Gold antifade reagent with DAPI (Molecular Probes, Invitrogen). For negative control, sections were incubated with either blocking buffer without primary antibody or with normal rabbit IgG.

RNA isolation from biopsies

Punch biopsies were transferred to 1 mL of -80°C cold RNALater-ICE (Ambion Inc., Austin, TX, USA) and kept at -80°C. 24 hours before purification the biopsies were transferred to -20°C. Upon RNA purification, biopsies were removed from RNALater-ice to 175 μL SV RNA lysis buffer (added β-mercaptoethanol and homogenized. RNA purification, including DNase treatment, was completed according to the manufacturer’s instructions (SV Total RNA isolation Systems; Promega, Madison, WI, USA). The RNA was stored at -80°C until further use.

DNA isolation from cell cultures

The cell supernatant was discarded and while on ice the keratinocytes were washed once in ice-cold sterile phosphate buffered saline (Gibco/Invitrogen, Carlsbad, CA, USA). 150 μL RNA cell lysis buffer were added and the prepared lysis transferred to eppendorf tubes and kept at -80°C until RNA purification. The purification was completed according to manufacturer’s instructions (SV 96 Total RNA isolation Systems; Madison, WI, USA) RNA was stored at -80°C until further use.

Western Blotting

The protein extract was prepared from keratinocyte biopsies. Biopsies were homogenized in a cell lysis buffer (20 mM Tris-base (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na3VO4, 1 μg mL-1 leupeptin and 1 mM PMSF) and left on ice for 30 minutes. The samples were centrifuged at 10,000 x g for 10 minutes at 4°C, after which the supernatant constituted the cell lysate.

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Statistical analysis

For statistical analysis a Student’s t-test (two-tailed) was performed. P<0.05 was regarded as statistically significant. The results are expressed as mean ± standard deviation.

Results

14-3-3 mRNA expression profiles in psoriasis, atopic dermatitis, allergic contact dermatitis and basal cell carcinoma

To investigate the mRNA expression profile of the 14-3-3 family members in psoriasis, atopic dermatitis, allergic contact dermatitis, and basal cell carcinoma, paired punch biopsies were obtained from involved and uninvolved skin. Total RNA was isolated and analysed by qRT-PCR. 14-3-3β, γ, ε, ζ, σ, and τ mRNA expression levels were significantly increased in involved psoriatic skin compared with uninvolved psoriatic skin (n=9) (Figure 1A). The 14-3-3σ and -τ mRNA levels were 2-fold increased whereas 14-3-3β, γ, ε and ζ mRNA levels were less than 2-fold increased in involved psoriatic skin, compared with paired tissue samples of uninvolved psoriatic skin. No significant changes were seen for 14-3-3η mRNA expression in psoriatic skin.

In biopsies from basal cell carcinomas (n=3) a significant 1.6-fold increase in 14-3-3ε mRNA expression was seen when compared with biopsies from normal skin from the patients (Figure 1B). 14-3-3τ mRNA expression was increased 2.4 fold (P=0.052) in biopsies from basal cell carcinomas. No significant changes were seen in mRNA levels of any of the other isoforms.

Figure 1C shows the mRNA level of the various 14-3-3 isoforms in three patients with atopic dermatitis (n=3). Although some variation in mRNA expression was found, no statistically significant differences were seen between involved and uninvolved atopic skin for any of the investigated 14-3-3 isoforms.

The results from three patients with nickel-induced contact dermatitis are displayed in figure 1D. Biopsies were collected 72 hours after patch testing with nickel. Analysis showed a significant 2.7-fold increase in 14-3-3τ and -σ mRNA expression in involved skin compared with uninvolved skin. 14-3-3 β, γ and -η mRNA levels were also increased in involved skin, but the differences were not statistically significant.

14-3-3 protein expression in psoriatic skin

To assess 14-3-3 isoform protein expression levels by Western blotting technique, keratome biopsies from nine psoriasis patients were collected. It was not feasible to collect keratome biopsies from patients with BCC, atopic eczema or contact dermatitis. The protein expression in involved psoriatic skin was compared with paired samples of uninvolved psoriatic skin. 14-3-3τ was the only isoform showing a consistent and significantly increased expression in involved psoriatic skin compared with uninvolved psoriatic skin. On the other hand, 14-3-3ε protein expression was slightly, but significantly (P<0.05), decreased in involved psoriatic skin. No significant changes in the protein expression in psoriatic skin were observed for the other 14-3-3 isoforms (Figure 2A-B).
**Immunofluorescence of 14-3-3τ and 14-3-3σ**

Because the mRNA expression of 14-3-3τ and 14-3-3σ was significantly upregulated by more than two fold in involved skin from both psoriasis and nickel induced contact dermatitis and a marked although statistically insignificant upregulation of 14-3-3τ was seen in BCC’s, the localization of 14-3-3τ and 14-3-3σ was examined by immunofluorescence in involved and uninvolved tissue sections from these diseases. Both 14-3-3τ and 14-3-3σ showed solid staining of the whole epidermis and higher magnification revealed localization of the proteins to the cytoplasm of the keratinocytes. The distribution of 14-3-3τ (Figure 3A-F) as well as 14-3-3σ (Figure 4A-F) was similar in involved and uninvolved skin in all investigated skin diseases.

**Regulation of 14-3-3τ expression in cultured normal human keratinocytes**

The mechanisms leading to increased 14-3-3τ mRNA expression in psoriasis, BCC, and allergic contact dermatitis are unknown. In an attempt to identify stimuli resulting in increased 14-3-3τ mRNA expression, *in vitro* experiments were conducted with cultured normal human keratinocytes. Keratinocytes were stimulated with TNF-α, IFN-γ, IL-1β, TPA or anisomycin, respectively, for 3, 6, 12, and 24 hours. Subsequently 14-3-3τ mRNA levels were analyzed using qRT-PCR. No regulation of 14-3-3τ mRNA expression was seen for any of the stimuli investigated (Figure 5A). When keratinocyte differentiation was induced by calcium (0.3 mM) the 14-3-3τ mRNA level increased. At 24 hours, 14-3-3τ mRNA was increased 1.3-fold compared with medium controls (P<0.05). At 48 hours, increased calcium concentrations resulted in a 1.4-fold increase in 14-3-3τ mRNA. However, at this time point the increase was not statistically significant compared with medium controls (Figure 5B).

**Discussion**

14-3-3 isoforms are involved in the regulation of numerous cellular processes, but their expression in common skin disease has not previously been studied. The present study demonstrates a significant 2-fold increase in the mRNA expression of 14-3-3τ and -σ in involved psoriatic skin and in involved skin from nickel-induced allergic contact dermatitis compared with paired samples of uninvolved skin. In basal cell carcinomas only 14-3-3τ mRNA expression was increased more than 2-fold, whereas the 14-3-3σ mRNA level were slightly, although not significantly, decreased.

In involved and uninvolved atopic dermatitis skin no significant differences in the mRNA expression of the various isoforms could be detected. 14-3-3 protein expression was only studied in tissue samples from psoriasis patients and only the expression of the 14-3-3τ protein was significantly increased in involved psoriatic skin compared with uninvolved psoriatic skin.

14-3-3 protein is involved in the regulation of transcription and translation by interacting with DNA/mRNA-binding proteins. Tristetraprolin (TTP) is a mRNA regulating protein, which through binding to AU-rich elements in cytokine mRNA (e.g. TNF-α), induces mRNA destabilization and subsequently degradation. MAPK-activated protein kinase 2 (MK2)-induced phosphorylation of TTP creates a...

**Figure 3. Immunofluorescence staining with 14-3-3τ.** Localisation of 14-3-3τ in the epidermis and dermis was analysed with immunofluorescence staining. Nuclear staining was performed using DAPI (blue). Green (Alexa Fluor 488) demonstrates 14-3-3τ. The following paired tissue sections were stained: (A) uninvolved psoriatic skin, (B) involved psoriatic skin, (C) uninvolved BCC, (D) involved BCC, (E) uninvolved allergic contact dermatitis, (F) involved allergic contact dermatitis.

**Figure 4. Immunofluorescence staining with 14-3-3σ.** Localisation of 14-3-3σ in the epidermis and dermis was analysed with immunofluorescence staining. Nuclear staining was performed using DAPI (blue). Green (Alexa Fluor 488) demonstrates 14-3-3σ. The following paired tissue sections were stained: (A) uninvolved psoriatic skin, (B) involved psoriatic skin, (C) uninvolved BCC, (D) involved BCC, (E) uninvolved allergic contact dermatitis, (F) involved allergic contact dermatitis.
functional binding site for 14-3-3. When 14-3-3 binds to TTP, the mRNA-degrading capabilities of TTP is inhibited. A previous work from our group demonstrated increased MK2 activation in involved psoriatic skin compared with uninvolved skin. The protein expression of TNF-α was also elevated in psoriatic skin compared with uninvolved skin, whereas the TNF-α mRNA level was similar, indicating a posttranscriptional regulation of TNF-α expression in psoriasis and MK2 was suggested to play a role in this posttranscriptional regulation.21 It is possible that MK2 regulates TNF-α expression through TTP and 14-3-3. Our unpublished results showed no regulation of TTP mRNA and protein expression in involved psoriatic skin compared with uninvolved psoriatic skin (data not shown). Instead this study showed that the mRNA expression of six isoforms of 14-3-3 was elevated in involved psoriatic skin compared with uninvolved skin, but only 14-3-3 was significantly elevated at the protein level. The precise role of 14-3-3 in the regulation of TNF-α expression in psoriasis is not yet known. It is intriguing to speculate that an increased activity of MK2 combined with elevated protein expression of 14-3-3 inhibits the destabilising effect of TTP on TNF-α mRNA thereby promoting TTP protein expression, but further studies are needed to clarify this.

Powell et al. demonstrated that MK2 phosphorylation 14-3-3ζ at Ser-58 compromises 14-3-3ζ dimerization. We found no regulation of 14-3-3ζ protein expression in involved psoriatic skin compared with uninvolved, but increased phosphorylation of 14-3-3ζ might affect its binding of TTP.

14-3-3ζ regulates the G2/M cell cycle checkpoint and DNA damage induces 14-3-3ζ protein expression in a p53 dependent manner. Loss of 14-3-3ζ increases the genomic instability in cells. Breast cancer cells lacking 14-3-3ζ expression show an increased number of chromosomal breaks and gaps when exposed to gamma-irradiation and 14-3-3ζ knockout cells have an increased frequency of chromosome aberrations. 14-3-3ζ can be inactivated by CpG-methylation (epigenetic silencing) of the promoter region. CpG methylation is a common event during carcinogenesis and has been reported in breast cancer, gastric, hepatocellular and small-cell carcinoma, oral squamous cell carcinoma, and basal cell carcinoma. Using immunohistochemistry Lodging et al. observed a strongly reduced 14-3-3ζ expression in basal cell carcinomas compared with normal skin, whereas the 14-3-3ζ expression level in lesional psoriatic skin was comparable to normal skin. Interestingly, we found no regulation of 14-3-3ζ mRNA expression in basal cell carcinomas, whereas an increased mRNA expression in involved psoriatic skin and in allergic contact dermatitis was detected in this study. Furthermore, a slight although statistically insignificant increase in 14-3-3ζ protein expression was seen in involved psoriatic skin compared with uninvolved skin. These differences may be explained by the different techniques used in the two studies. We detected mRNA using qRT-PCR whereas protein levels were detected by immunohistochemistry by Lodging et al. Maintaining or even increasing the expression of 14-3-3ζ in hyperproliferative skin disorders like psoriasis and allergic contact dermatitis may be a key control point in preventing malignant transformation by activating the G2/M checkpoint in the cell cycles.

Taken together, we showed an upregulation of 14-3-3ζ expression in psoriasis, allergic contact dermatitis and BCC whereas the expression of 14-3-3ζ was increased only in psoriasis and allergic contact dermatitis. Thus, functional studies of the individual 14-3-3 isoforms in keratinocytes are highly needed in order to increase our understanding of a possible role of the various 14-3-3 isoforms in the pathogenesis of both benign and malignant skin diseases.

### References

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