The expression and function of psoriasin in two tissue types in vitro

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Abstract

Psoriasin (S100A7) is a protein of low molecular weight that belongs to the S100 protein family. The S100 family is characterised by EF hand type domains important for their function as calcium-binding proteins. The expression of psoriasin has been found to be highly up-regulated in the benign hyperproliferative skin disorder psoriasis and has also been associated with some subtypes of breast cancer, including ductal carcinoma in situ (DCIS). Psoriasin is thereby over-expressed in abnormal conditions which share the following features: cellular and vascular hyperproliferation, abnormal differentiation and lymphocytic infiltration. Despite extensive research the specific function of psoriasin is at present unknown. The protein is localised in the nucleus and the cytoplasm but has also been reported to be secreted. This might suggest both an intracellular and an extracellular function. The purpose of this study was to examine the expression pattern and the function of psoriasin in keratinocytes and mammary epithelial cells in vitro. The expression pattern of psoriasin was examined using several substances which were tested for their ability to induce the expression and to inhibit an established expression. The psoriasin expression in keratinocytes was found to be induced by the loss of attachment to ECM, a factor which previously has been reported to up-regulate the expression in mammary epithelial cells. The expression was also successfully inhibited in mammary epithelial cells and keratinocytes by transfection with a recombinant adenovirus carrying the Bcl2-gene, a potent apoptotic inhibitor. The expression was inhibited regardless of the dose of Bcl2. The relationship between Bcl2 and psoriasin is thereby subject for further research. The second purpose of this study was to investigate the influence of psoriasin on proliferation, sensitivity to apoptosis, differentiation and capability of invasion. The influence was studied using several functional in vitro assays. At present several experiments studying the expression and function of psoriasin are in progress. These interesting approaches will hopefully in a near future lead to the establishment of the function for psoriasin.
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1. Introduction

The protein psoriasin (S100A7) was originally identified in 1991 as a protein of low molecular weight, approximately 11kDa, which is highly up-regulated in psoriatic keratinocytes [Madsen et al 1991]. Psoriasin is a calcium-binding protein and contains all the sequence features conserved in the S100 gene family [Hoffmann et al 1994]. The S100 gene family encodes small cytoplasmatic and secreted proteins and is characterised by the EF-hand helix-loop-helix domains important for the function as calcium-binding proteins. The S100 proteins have been proposed to function in cell differentiation, cell cycle progression, energy metabolism, kinase activity and cytoskeletal membrane interactions [Donato 1999]. Abnormal expression of these proteins has also been associated with disease conditions. The chromosomal position for several members of the S100 gene family is 1q21 where the psoriasin gene is located as well. The psoriasin gene comprises three exons and two introns (GenBank AJ012825) and encodes a protein of 101 amino acids. Mutational analysis of the coding region in psoriatic patients has not revealed any nucleotide variants and the protein expressed is thus identical in healthy and non-healthy individuals [Semprini et al 1999]. The expression of psoriasin has been reported to be localised within both the nucleus as well as the cytoplasm in breast epithelial tumour cells and keratinocytes [Al-Haddad et al 1999]. Additionally, psoriasin has also been reported to be secreted [Celis JE et al 1996]. This might suggest both an intracellular and an extracellular function. The specific function of psoriasin has yet to be established although a possible function as a chemotactic protein for CD4+ T-lymphocytes and neutrophils has been reported [Jinquan et al 1996]. A number of interaction partners for psoriasin such as E-FABP [Hagens et al 1999], RanBPM [Emberely et al 2002] and JABP [Emberely et al 2003] have been proposed but the consequence of these findings remains to be fully elucidated.

Psoriasin exhibits restricted occurrence in normal tissues. For example normal skin shows very low mRNA levels while other human tissues do not display any detectable levels [Madsen et al 1991]. Neither do a wide range of human cell types express psoriasin under normal conditions. Psoriasin is as mentioned above abundant in psoriatic keratinocytes and has also been found in abnormally differentiating keratinocytes in vitro [Madsen et al 1991] and in a variety of inflammatory skin disorders including atopic dermatitis, mycosis fungoides, Darier's disease and lichen sclerosus et atrophicus [Algermissen et al 1996].
addition to various skin lesions up-regulated psoriasin mRNA expression is also found in squamous carcinoma of the skin [Alowami et al 2003] and squamous cell carcinoma of the bladder [Ostergaard et al 1999]. This particular subtype of bladder cancer show resemblance to epidermis and the cells involved in these skin and cancer lesions are thereby similar. Over-expression of psoriasin has also been associated with a variety of breast carcinomas [Moog-Lutz et al 1995] and with gastric carcinoma although only in a fraction of the samples examined [El-Rifai et al 2002]. Psoriasin is thus involved in a number of abnormal conditions which share the following features: cellular and vascular hyperproliferation together with abnormal differentiation and lymphocytic infiltration. The association with cancer is very interesting and emphasises the importance of identifying the function of the protein.

The malignant transformation of a normal cell is a dynamic process with sequential multistep evolution [Beckmann et al 1997] characterised by accumulation of genetic alterations. All types of human cancer cells have acquired capabilities enabling the transformation of the normal cell into a cancer cell. The cancer cells have defects in the regulatory pathways concerning proliferation and homeostasis. One has suggested six essential alterations which collectively rule the transformation [Hanahan and Weinberg 2000]. These are self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. Cancer develops thus in general through transformation of normal cells via hyperplasia, premalignant change, in situ carcinoma and invasive tumour.

Breast cancer is the most common form of cancer in women and represents a variety of tumours with different cell origin. The number of breast cancer cases has lately increased due to the extension and the high sensitivity of mammography. One-fifth of all mammographically detected breast cancer is today represented by ductal carcinoma in situ, DCIS [Mokbel 2003]. DCIS is a heterogeneous cancer type characterised by proliferation of malignant epithelial cells that are confined within the basement membrane of the mammary ducts. Various subtypes of DCIS have been described and at present it is unknown which subtypes of DCIS will transform into an invasive form. DCIS can be considered as an early breast cancer lesion and has not acquired the sixth capability described above. Through the tumour progression genetic alterations accumulate and random alterations become more and more abundant. To be able to elucidate the pathways behind the progression it is necessary to
focus on those events of real importance for the progression. In order to avoid studying random events the early lesion of DCIS is a suitable investigative material.

Through SAGE analysis psoriasin has been shown to be one of the most abundant transcripts in high-grade DCIS [Enerbäck et al 2002]. Additionally, FISH was performed to confirm that the aberrant expression was not caused by amplification of the psoriasin locus on chromosome 1q. Analysis of in situ and invasive components of the same breast tumour revealed a higher expression of the mRNA transcript encoding psoriasin in the former of the two breast components. The finding was further supported by similar analysis performed on RNA extracted from independent breast samples representing a range from normal to invasive tumour tissues. High psoriasin expression was confirmed in the in situ tumour only while normal, benign and invasive tumour tissues showed low or no detectable levels [Leygue et al 1996]. Moreover, another group has examined the psoriasin mRNA levels in a cohort of invasive breast tumours. It was found that mRNA was detected in all tumours by RT-PCR but the levels varied considerably and were mostly low. Persistence of psoriasin at high levels in the invasive ductal tumour cells exhibited significantly correlation with poor prognosis, such as lack of the oestrogen and progesterone receptors. The psoriasin expression was also associated with inflammatory infiltrates [Al-Haddad et al 1999].

In vitro studies of up-regulation of psoriasin protein have been performed on normal mammary epithelial cells, MCF10A [Enerbäck et al 2002]. Increased expression was induced by loss of attachment to ECM, growth factor deprivation and confluent conditions. These conditions are representative for psoriasis as well as the tumour environment and consequently the mechanisms responsible for the abnormal expression may be similar in vitro and in vivo. The observation that the over-expression of psoriasin is found in the early form of breast tumours could indicate a participating roll for psoriasin in the tumour progression. On the other hand, psoriasin is over-expressed in psoriasis which is a completely benign hyper proliferation. It is therefore more likely that the protein might exhibit a protective function rather than being the cause of the malignancy process. The loss of psoriasin in breast tumours could therefore contribute to the onset of successful invasion [Watson et al 1998]. Further support for the roll as a participant in tumour progression is provided by its chromosomalous location on chromosome 1. This region frequently (>50%) exhibits loss of heterozygosity in invasive tumours [Munn et al 1995].
A study of psoriasin expression during skin tumour genesis showed compatible results with the observations in breast tumour genesis. Altered psoriasin expression with low or no expression was found in normal and invasive tumour tissue while the highest expression was observed in squamous carcinoma in situ [Alowami et al 2003]. Summarising these facts leads to an appealing thought that psoriasin could participate as a genetic factor with influence in the transition from benign to malign transformation.
2. Aim

The purpose of this study was to characterise the expression pattern and to investigate the biological function of psoriasin in two tissues, keratinocytes and mammary epithelial cells, in vitro. The expression pattern was studied using two different approaches. Several substances relevant for the pathology of the diseases were tested for their ability to induce the expression. Once this over-expression was established various substances were tested for their ability to inhibit the expression. The changes in expression were visualised by Western Blot.

The next aim was to study the function of the over-expressed protein and four functional systems were used. The influence of psoriasin on proliferation, sensitivity to apoptosis, differentiation process and capability of invasion was studied. These features are characteristic for both psoriatic and cancer lesions, conditions in which psoriasin are strongly over-expressed. In order to find a function for psoriasin relevant for cancer tumours as well as psoriasis the influences were investigated through several in vitro methods which mimic the environment shared by these two abnormal conditions. In all methods used the psoriasin-expressing cells were compared to non-expressing cells and the differences between the cell types were observed. The first functional approach included visual observations of the proliferation rate. The same procedure was used when the influence of psoriasin on the sensitivity to apoptosis was examined. The cells were exposed to four different environments with a substantial apoptotic potential. The environments included malnutrition, loss of attachment to ECM, over-expression of an apoptotic inducer (p53) and production of ROS (reactive oxygen species). The third functional approach was performed using RT-PCR to investigate the involvement of psoriasin on the differentiation process. RT-PCR was performed to detect expression changes of differentiation markers, in this case keratin 1 and 10, on the mRNA level. The final functional approach was to examine the influence of psoriasin on the capability of invasion. This was performed using an invasion chamber assay in which the cells are allowed to invade an in vitro extracellular matrix system by chemotaxis.
3. Material and methods

3.1 Cell lines

The cell lines used are briefly described in table 1. All cell lines were cultured in a humidified tissue culture incubator at 37°C, 5% CO₂ in media according to the supplier’s recommendations.

Table 1. Description of the cell lines used.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEKn</td>
<td>Primary human neonatal keratinocytes.</td>
</tr>
<tr>
<td>HaCaT</td>
<td>Spontaneously immortalised human keratinocytes.</td>
</tr>
<tr>
<td>MCF10AQ</td>
<td>Normal human mammary epithelial cells transfected by retrovirus without a gene (empty vector).</td>
</tr>
<tr>
<td>MCF10AHID</td>
<td>Normal human mammary epithelial cells transfected by retrovirus expressing HID5/psoriasin.</td>
</tr>
<tr>
<td>MCF7</td>
<td>Human mammary epithelial cancer cells.</td>
</tr>
<tr>
<td>MB435</td>
<td>Human mammary epithelial cancer cells.</td>
</tr>
<tr>
<td>MB468</td>
<td>Human mammary epithelial cancer cells expressing HID5/psoriasin.</td>
</tr>
<tr>
<td>TAC2tet</td>
<td>Mouse mammary epithelial cells expressing HID5/psoriasin in the absence of doxycycline.</td>
</tr>
</tbody>
</table>

3.2 Recombinant adenovirus

The viruses used are all recombinant adenovirus designed according to the AdEasy procedure. Beside the GFP-gene the viruses carry the following genes: p53, Bcl2, ErbB2 and HID5/psoriasin, respectively. The amount of virus needed for proper infection of the cell lines was also determined. The cell lines described above were plated at a density of 12x10⁴ cells/well in a 6-well plate. After attachment (next day) the cells were transfected with different amounts of virus and 24 hours later the infection was observed in fluorescence microscope (figure 1). It is of great importance to identify the correct amount of virus since a too low dose does not affect the cells and a too high dose may have a negative effect on the cells.

Figure 1.
Cells transfected with recombinant adenovirus expressing the GFP-gene.
3.3 Expression: Induction and inhibited induction

The inductions were made using HaCaT, HEKn and MCF10AQ. Stimuli relevant for induction experiments were selected due to their association to the features shared by psoriatic and cancer lesions (see table 2). Cells were cultured in $\varnothing 100$mm cell culture dishes at a density of $120 \times 10^4$ cells/dish in media according to the supplier’s recommendations. Additionally, $120 \times 10^4$ cells were centrifuged at 2500 rpm for three minutes. The cell pellet was immediately frozen in liquid nitrogen and used as a control. After attachment of the cells the substances were added. The time of exposure varied from 3 to 72 hours. The substances, concentrations and exposure times are summarised in table 2. After incubation the cells were harvested by scraping, centrifuged at 2500 rpm for three minutes and the cell pellets were immediately frozen in liquid nitrogen. Analysis was made by Western Blot (3.3.1).

Table 2. Induction: Substances, concentrations and exposure times.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Function</th>
<th>Concentration</th>
<th>Exposure time</th>
</tr>
</thead>
<tbody>
<tr>
<td>poly-2-hydroxy-ethylmethacrylate</td>
<td>Inhibits cell attachment to the</td>
<td>10mg/cm²</td>
<td>24, 48 and 72 h</td>
</tr>
<tr>
<td>(polyHEME)</td>
<td>culture dishes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL1α</td>
<td>Cytokine important in psoriasis</td>
<td>20ng/ml</td>
<td>6, 24 and 72 h</td>
</tr>
<tr>
<td>IL2</td>
<td>Cytokine important in psoriasis</td>
<td>0.005µg/ml</td>
<td>6 and 24 h</td>
</tr>
<tr>
<td>INFγ</td>
<td>Cytokine important in psoriasis</td>
<td>0.04µg/ml</td>
<td>6 and 24 h</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>Inhibitor of the PI-kinase pathway</td>
<td>1µM</td>
<td>6 and 24 h</td>
</tr>
<tr>
<td>Phorbolester TPA</td>
<td>Carcinogen</td>
<td>0.01mg/ml</td>
<td>6 and 24 h</td>
</tr>
<tr>
<td>Cyclosporine A</td>
<td>Immunosuppressor which inhibits the</td>
<td>1µM</td>
<td>72 h</td>
</tr>
<tr>
<td></td>
<td>cytokine production, used as treatment for psoriasis</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Inhibited induction experiments were performed primarily on MCF10AQ but also on HaCaT and HEKn. Cells were plated in poly-2-hydroxy-ethylmethacrylate (polyHEME,10mg/cm²)-coated $\varnothing 60$mm cell culture dishes at a density of $120 \times 10^4$ cells/dish in media recommended by the supplier. The polyHEME prevents the attachment of the cells and the cells are cultured in suspension, a condition which induces the over-expression of psoriasin. Additionally, $120 \times 10^4$ cells were centrifuged at 2500 rpm for three minutes. The cell pellet was immediately frozen in liquid nitrogen and used as a control. The substances (summarised in table 3) were added and the cells were incubated for 48 hours (72 hours for HaCaT and HEKn). After incubation the cells were collected and centrifuged at 2500 rpm for three minutes. The cell pellets were immediately frozen in liquid nitrogen. Analysis was made by Western Blot (3.3.1).
Table 3. Substances used for inhibited induction, concentrations and exposure times.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Function</th>
<th>Concentration</th>
<th>Exposure time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl2-virus</td>
<td>Apoptosis inhibitor</td>
<td>1µl to 7 ml of media</td>
<td>48 and 72 h</td>
</tr>
<tr>
<td>p53-virus</td>
<td>Apoptosis inducer</td>
<td>1µl to 7 ml of media</td>
<td>48 and 72 h</td>
</tr>
<tr>
<td>Metotrexate</td>
<td>Folic acid antagonist, used as treatment of psoriasis</td>
<td>2.5µM</td>
<td>48 h</td>
</tr>
<tr>
<td>Cyclosporine A</td>
<td>Immunosuppressor which inhibits the cytokine production, used as treatment for psoriasis</td>
<td>0.1µM</td>
<td>48 and 72h</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>Immunosuppressor, used as treatment of psoriasis</td>
<td>10µg/ml</td>
<td>48 h</td>
</tr>
<tr>
<td>INFγ</td>
<td>Cytokine important in psoriasis</td>
<td>0.04µg/ml</td>
<td>48 h</td>
</tr>
<tr>
<td>Insulin</td>
<td>Stimulates the uptake of glucose and the synthesis of protein and lipids</td>
<td>20µg/ml</td>
<td>48 h</td>
</tr>
<tr>
<td>Control (only polyHEME)</td>
<td>See table 2</td>
<td></td>
<td>48 and 72 h</td>
</tr>
</tbody>
</table>

3.3.1 Western Blot

The protein samples stored in -70°C were thawed on ice and one volume of DTT-hypotone buffer was added. The samples were sonicated and diluted in one volume of sample buffer containing β-mercaptoethanol. The samples were denaturated at 95°C for 5 minutes and loaded on a NuPAGE™ 4-12% Bis-Tris Gel (Invitrogen) together with 20µl of MultiMark® Multi-Colored Standard ladder (Invitrogen). The gel was run in 1x NuPAGE™ MES SDS Running Buffer (Invitrogen) at 200V for about one hour. Two Whatman filters and a 140µm nitrocellulose blotting membrane (PALL) were moistened in 1x NuPAGE™ Transfer buffer. Following electrophoresis the gel was placed on one of the Whatman filters with the nitrocellulose membrane and the other Whatman filter on top in a blot module. The blot was performed at 20V for two hours in 1x NuPAGE™ Transfer buffer. The membrane was blocked in 5% skimmed milk powder in Tris-buffered saline-0.05% Tween (1xTBST-buffer) over night. The next morning the membrane was cut in two where the upper half was incubated with primary monoclonal tubulin antibody while the lower half was incubated with primary HID5/psoriasin polyclonal antibody for one hour on a Belly Dancer. Both of the antibodies were diluted 1:10 000 in 5% skimmed milk powder in 1xTBST-buffer. The amount of detected tubulin is important and represents equal loading on the gel. The membranes were washed in 1xTBST for 15 minutes followed by incubation with secondary antibodies. Horse radish peroxidase-conjugated AffiniPure Goat Anti-Mouse (tubulin) or Anti-rabbit (HID5/psoriasin) IgG (Jackson ImmunoResearch) served as secondary antibodies. The membranes were washed three times ten minutes in 1xTBST and visualised after incubation in 2 ml of SuperSignal®Stable Peroxide Solution together with 2 ml of SuperSignal®West Pico Luminol/Enhancer Solution (Pierce) for five minutes. The SuperSignal® solutions are
chemiluminescent substrates. The horse radish peroxidase conjugated to the secondary antibodies reacts with the substrates and the chemiluminescence signal is registered with a FluorChem camera.

3.4 Proliferation

Proliferation experiments were performed using MCF7 and TAC2tet cell lines. MCF7 was cultured in $\varnothing$100 mm cell culture dishes at a density of $50 \times 10^4$ cells/dish. After attachment of the cells four dishes were infected with $4 \mu l$ of GFP-virus, $4 \mu l$ of HID5/psoriasin-virus, $4 \mu l$ of ErbB2-virus and $2 \mu l$ of HID5-virus together with $2 \mu l$ of ErbB2-virus, respectively. The amount of virus needed for infection had previously been determined by observing cells transfected with various amounts of virus in fluorescence microscope (see 3.2). Infection with GFP-virus is performed to be able to exclude the risk that the virus itself affects the proliferation and serves thus as a control. The media volume was adjusted to 5 ml to achieve efficient infection. 48 hours after the virus infection the transfected cells and one control dish were treated with $1 \mu M$ of ionophore (A23187) and incubated at $37^\circ C$ for 30 minutes. The ionophore is a small hydrophobic mobile ion carrier which increases the ion permeability in lipid bilayers. It increases the intracellular level of $Ca^{2+}$ which might be needed for the proper function of the over-expressed psoriasin. Following the ionophore treatment the cells were incubated in $37^\circ C$ and the proliferation was observed during a couple of days before staining with crystal violet. The cell culture dishes were washed in phosphate buffered saline, PBS, followed by staining in crystal violet for 20 minutes. The stained dishes were washed with additional PBS three times and were thereafter air-dried. The four transfected and ionophore treated cell cultures were compared to controls with and without ionophore treatment. This experiment was also performed on MB435, strictly according to the procedure described above.

Additionally, the proliferation study was performed on the TAC2tet cell line. The TAC2tet cell line is a transformed cell line which expresses psoriasin in the absence of doxycycline (tetracycline). TAC2tet was plated at a density of $120 \times 10^4$ cells/dish in four $\varnothing$100 mm collagen-coated cell culture dishes. After attachment of the cells the media was exchanged to media without doxycycline in two of the four culture dishes and the expression of psoriasin was thus activated. 24 hours after the change of media two cell culture dishes with and without doxycycline, respectively, were treated with $1 \mu M$ of ionophore for 30 minutes. After
ionophore treatment media was exchanged in all cell culture dishes and the proliferation was observed during a couple of days before staining with crystal violet (see procedure described above).

### 3.5 Sensitivity to apoptosis
In order to study if psoriasin has influence on the sensitivity to apoptosis psoriasin-expressing and non-expressing cells were exposed to four different apoptotic conditions. The first approach was to culture cells in serum free media in polyHHEMA-(10mg/cm²) coated cell culture dishes during 24 and 48 hours. The polyHHEMA prevents attachment of the cells to the culture dish and mimics the condition where cells lose contact with the extracellular matrix, a feature shared by cancer cells and psoriatic cells. The interaction with ECM is essential for the normal function of the cell and the polyHHEMA could therefore be classified as an apoptotic inducer. The serum free media causes malnutrition and culturing in polyHHEMA together with serum free media creates a potent apoptotic environment. Another approach of mimicking an apoptotic environment is to infect the cells with recombinant virus carrying the p53-gene which leads to an over-expression of the apoptotic inducer p53. In the fourth and final approach cells were exposed to menadione in various concentrations. Menadione induces the production of ROS (reactive oxygen species) which in turn induce apoptosis. Cells were also cultured with high extracellular Ca²⁺ levels. In one of the experiments the cells were exposed to ionophore. The ionophore enables as mentioned earlier the extracellular calcium ions to enter the cell and may thereby interact with the intracellular psoriasin.

The cell lines used for this study were MCF10AQ, MCF10AHID and HaCaT. Each cell line was plated in Ø100 mm cell culture dishes at a density of 80x10⁴ cells/dish. The next morning two HaCaT cell cultures were transfected with 15µl of HID5/psoriasin-virus and another two were transfected with 4µl of GFP-virus. The media volume was adjusted to 5 ml to ensure proper infection. 24 hours after infection one HaCaT culture transfected with HID5-virus and one transfected with GFP-virus were trypsinised and transferred to polyHHEMA-(10mg/cm²) coated Ø60 mm plates containing serum free media. The remaining two HaCaT plates transfected with HID5/psoriasin-virus and GFP-virus served as controls and the media was therefore exchanged to serum free media. Two dishes of MCF10AQ and MCF10AHID, respectively, were trypsinised and transferred to polyHHEMA-(10mg/cm²) coated Ø60 mm plates containing serum free media. Media was exchanged to serum free media in another two
dishes of MCF10AQ and MCF10AHID, respectively, and these served as controls. The cells were cultured in the polyHEME plates for 24 hours (MCF10A) and 48 hours (MCF10A and HaCaT). After the polyHEME treatment the cells were harvested by trypsinisation and 0.5% of the each cell culture was transferred to T25 cell culture flasks. The cells were cultured in a tissue culture incubator for a couple of days before staining in crystal violet for 20 minutes (see staining procedure part 3.4). The effect of psoriasin on proliferation rate/apoptotic sensitivity was evaluated by comparing the psoriasin-expressing and the non-expressing cells.

In the second approach MCF10AQ and MCF10AHID were plated at a density of 120x10⁴ cells/dish in media containing 2mM CaCl₂. After attachment the following morning the cells were transfected with 4µl of p53-virus and 2µl of GFP-virus. Dishes infected with 2µl of GFP-virus served as controls. The media volume was adjusted to 5 ml to optimise the infection. The cells were then incubated for 72 hours and harvested according to the procedure described above.

MCF10AQ and MCF10AHID were also plated at a density of 120x10⁴ cells/dish in media containing 1.5mM CaCl₂. After attachment the cells were treated with 3µM ionophore for one hour. The media was then exchanged and menadione was added at concentrations of 40, 50 and 60µM. Cells exposed to menadione 40µM was incubated for 24 hours while cells cultured in menadione 50 and 60µM were incubated for 2 hours. The cells were harvested according to the procedure described above.

3.6 Differentiation

The influence of psoriasin on differentiation was analysed by RT-PCR in HaCaT. In HaCaT the markers for differentiation were represented by keratin 1 and 10. In order to induce differentiation and create a positive control HaCaT were cultured in suspension for 24 hours using polyHEME-(10mg/cm²) coated plates. The culturing in polyHEME for 24 hours induces differentiation and up-regulates both keratin 1 and 10. HaCaT cultured under normal conditions was used as a negative control. Two main questions constituted the basis of this experiment: Do psoriasin affect the differentiation process? Is psoriasin capable to induce the differentiation process? To be able to investigate if psoriasin affects the differentiation process differentiation was induced (see method below) in cells transfected with HID5/psoriasin-and GFP-virus. RNA was prepared from the different cells and any differences in the expression of keratin 1 and 10 between the psoriasin-expressing and the
non-expressing cells were analysed by RT-PCR. The PCR products were separated and visualised by gel electrophoresis.

HaCaT were plated in Ø100 mm cell culture dishes at a density of 120x10⁴ cells/dish. Next morning the cells were transfected with 10μl of HID5-virus and 5 µl of GFP-virus, respectively. 24 hours after infection the cells were treated with 1µM of ionophore for 30 minutes. Differentiation were then induced either by culturing the transfected cells in 1µM wortmannin for 24 hours [Sayama et al 2002], 4mM CaCl₂ for 48 hours or by culturing in suspension using polyHEME-(10mg/cm²) coated plates for 24 hours. After the incubation cells were harvested by scraping, centrifuged at 2500 rpm for three minutes. The cell pellets were immediately frozen in liquid nitrogen before RNA preparation.

In order to investigate if the psoriasin is capable to induce the differentiation process HaCaT was plated in Ø100 mm cell culture dishes at a density of 120x10⁴ cells/dish. After attachment the cells were transfected with 10μl of HID5-virus and 5µl of GFP-virus, respectively. 24 hours after infection the cells were treated with 1µM of ionophore for 30 minutes. Media was then exchanged and the cells were incubated for 24 hours before harvesting described above. The cell pellets were immediately frozen in liquid nitrogen before RNA preparation.

3.6.1 RNA preparation
RNA preparation was performed with RNeasy® Mini Kit (Qiagen) according to the manufacturer’s protocol (see appendix). The RNA was eluted in RNase-free water and stored in -80°C. The total RNA concentration was determined by measurements of absorbance at 260 nm in a GeneQuant II RNA/DNA calculator (Pharmacia Biotech). The RNA was diluted in TE buffer pH 8.0, a buffer which also served as a reference.

3.6.2 cDNA synthesis
cDNA was synthesised using SuperScript™II RNase H⁻ Reverse Transcriptase (Invitrogen). 0,5μl of 500μg/ml Random primers, 1μl of 10mM dNTPs, approximately 5μg total RNA and sterilised dH₂O up to 12μl in an Eppendorf tube were incubated at 65°C for five minutes and chilled immediately thereafter on ice. 4μl of 5xFirst Strand Buffer, 2μl of 0,1M DTT and 1μl of 40u/μl RNasin (Promega) was added, gently mixed and incubated at 42°C for two minutes. 1μl of SuperScript™II was added and the contents of the Eppendorf tube were mixed by
pipetting up and down. The synthesis reaction was incubated at 25°C for 10 minutes and 42°C for 50 minutes. Finally the reaction was inactivated by incubation at 70°C for 15 minutes. The quality of the cDNA was verified by amplification of a β-actin fragment (478bp) by PCR. The β-actin primer sequences, forward 5’-cccagatcatgtttgagacc-3’ and reverse 5’-aaggtagttcatggtatgcc-3’, were kindly supplied from Dr Dale Porter at the Dana-Farber Cancer Institute in Boston. The PCR amplification was performed under following conditions: 1.5 mM MgCl₂, 0.5µM of each primer, 0.2mM dNTPs and 2.5 units of TaqGold. TaqGold was first activated at 94°C for 10 minutes followed by 25 cycles of denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute and extension at 72°C for 1 minute. Finally the whole reaction was terminated at 72°C for 10 minutes.

3.6.3 PCR

Published primer sequences [Pivarcsi et al 2001] were verified to both the mRNA sequence (GenBank NM_006121 and NM_000421 for keratin 1 and 10 respectively) and to the genomic sequence (GenBank AF304164 for keratin 1). The PCR reaction of total 20µl contained 10.3µl of sterilised dH₂O, 2µl of 10xPCR Buffer containing 15mM MgCl₂, 3.2µl of 10mM dNTPs, 1µl of 10µM F-primer, 1µl of 10µM R-primer, 2µl of 1:5 diluted cDNA and 2.5 units of TaqGold. The PCR amplification was the identical for both keratin 1 and 10 and consisted of an activation step for the TaqGold at 94°C for 10 minutes followed by 27 cycles of denaturation at 94°C for 90 seconds, annealing at 60°C for 90 seconds and extension at 72°C for 120 seconds. The PCR amplification products (317 bp for keratin 1 and 686 bp for keratin 10) were analysed by electrophoresis and run on a 2% agarose gel with ethidium bromide staining to visualise the products under UV illumination.

3.7 Capability of invasion

The effect of psoriasin on the invasion potential of the cell was analysed by a cell invasion assay using BD BioCoat™ Matrigel™ Invasion Chamber (BD Bioscience). The cell line used was MB435 and MCF10A. MB435 was plated at a density of 150x10⁴ cells/dish in three Ø100mm cell culture dishes and cultured in media according to the supplier’s recommendations. After attachment of the cells two of the dishes were infected with 3µl of HID5/psoriasin-virus and 3µl of GFP-virus, respectively. 24 hours after infection the cells were harvested and cell suspensions in serum free media at a concentration of 5x10⁴ cells/ml were prepared. Non-transfected MB435 served as a control. As a comparable experiment
MCF10AQ and MCF10AHID were prepared in serum free media at a concentration of $5 \times 10^4$ cells/ml. Each of the five cell types (MB435, MB435 transfected with GFP-virus, MB435 transfected with HID5-virus, MCF10AQ and MCF10AHID) were used in duplicate. Ten Matrigel inserts was thawed and rehydrated with 37°C serum free media containing 0.1% of BSA for two hours, 0.5 ml in the well and 0.5 ml in the insert. The five control inserts, one for each cell type, were treated likewise. 750µl of chemoattractant (serum containing media) was added to equal number of Matrigel and control wells. The inserts were carefully transferred from the rehydration wells to the wells containing chemoattractant. The rehydration media was removed from the inserts and the cell suspensions were immediately added to the inserts. The invasion chambers were thereafter incubated in a humidified tissue culture incubator at 37°C, 5% CO$_2$ for 24 hours. After incubation the non-invading cells were removed from the upper surface of the membrane by scrubbing with two to three cotton tipped swabs. The invading cells were stained with crystal violet for 20 minutes followed by washing in water. The inserts were air-dried and the membranes were thereafter removed from the inserts by a sharp scalpel cutting at the edge of the housing wall. The membranes were placed in oil on a microscope slide and analysed in an inverted microscope. Cells in five fields at high magnification chosen at random was counted in both Matrigel and control membranes. The percentage of invasion was calculated by the formula below.

\[
\text{Mean number of cells invading through Matrigel insert membrane} \\
\% \text{ Invasion} = \frac{\text{Mean number of cells invading through Matrigel insert membrane}}{\text{Mean number of cells invading through control insert membrane}} \times 100
\]
4. Results

4.1 Expression: Induction and inhibited induction

A large spectrum of substances including cytokines, signal transduction inhibitors, carcinogens and immunosuppressors were tested for their ability to induce the expression of psoriasin in vitro. The substances chosen are relevant for the disease environment in psoriasis and DCIS. So far up-regulated psoriasin expression was confirmed in keratinocytes in conditions such as culturing in suspensions using polyHEME-coated plates. The psoriasin expression was up-regulated after 24 hours but a substantial over-expression was observed after 48 and 72 hours (figure 2). Up-regulation of psoriasin in cells cultured in suspension was also observed in mammary epithelial cells, previously described by Enerbäck et al 2002.

Figure 2.
Western Blot: HaCaT cultured in cell suspension using polyHEME-coated cell culture dishes.
Upper row: tubulin; lower row: psoriasin

Thus, culturing cells in suspension using polyHEME-coated plates induces a substantial over-expression of psoriasin. Once this over-expression had been established various substances were tested for their ability to inhibit the expression. Intriguingly positive results were achieved when normal human mammary epithelial cells (MCF10AQ) cultured in suspension were transfected with recombinant adenovirus carrying the Bcl2-gene. The psoriasin expression was repeatedly decreased to low levels (figure 3 lane 6) compared to the positive control (figure 3 lane 3). Detection of the tubulin amount of the cells (upper half of figure 3) represents equal loading of the samples on the gel.
As a follow-up, MCF10AQ was also cultured in suspension using polyHEME-coated plates and transfected with various doses of adenovirus carrying the Bcl2-gene. Various doses were used to investigate a possible dose-dependent influence of Bcl2. Doses of virus from 0.5µl up to 4µl were used (figure 4).

As figure 4 shows the amount of tubulin is not equal in the samples. In the highest dose of virus (4µl, figure 4 lane 7) the tubulin amount is very low indicating a reduction in the number of cells during the infection. The higher dose of virus the lower number of living cells in the samples. The variation in tubulin amount is thus due to the harmful effect of the virus. Additionally, the experiment was repeated with doses of virus lower than 1µl. The hazardous influence of the virus itself was thereby eliminated. This time the doses of virus used represented an interval from 0.01µl to 0.5µl (figure 5).
The expression of the Bcl2-gene was capable of inhibiting the psoriasin expression in all of the doses examined. The tubulin gives a strong signal in all of the lanes which indicates an equal loading of the samples. A very low expression of psoriasin was observed in the lowest dose of adenovirus (figure 5 lane 9).

To verify a general inhibition potential of Bcl2 on the psoriasin expression the experiment was performed in kertinocytes represented by HaCaT and HEKn (figure 6).

The inhibition was observed in HEKn after 72 hours (figure 6 lane 10). HaCaT transfected with Bcl2-adenovirus did not show any inhibition (figure 6 lane 6).
4.2 Proliferation
Ionophore-treated MCF7 cells transfected with HID5/psoriasin-virus, ErbB2-virus and HID5/psoriasin-virus together with ErbB2-virus were compared to the controls comprising untreated MCF7, MCF7 treated with ionophore and MCF7 transfected with GFP-virus. There was no difference in the proliferation rate between psoriasin-expressing cells and the controls. Identical results were shown in the MB435 and TAC2tet cell lines.

4.3 Sensitivity to apoptosis
Psoriasin-expressing and non-expressing normal human mammary epithelial cells and keratinocytes were exposed to four different environments which induce the process of apoptosis. The cells were exposed to over-expression of the apoptotic inducer p53, loss of attachment to ECM, malnutrition and production of reactive oxygen species. The proliferation was observed and visual interpretation has so far not revealed any differences in the sensitivity to apoptosis between psoriasin-expressing and non-expressing cells. The same results were achieved in both cell lines and in all four of the environments with a substantial apoptotic potential.

4.4 Differentiation
The quality of the cDNA synthesised was verified by amplification of β-actin fragments as described earlier. All of the cDNA synthesised showed distinct bands (data not shown) and the RNA preparation and cDNA synthesis had thus been successful. The results from the differentiation experiments are at present incomplete since the priority has not been placed on this approach. Functional primers, amplification protocols and cDNA of high quality are however prepared and material is thus ready for use. For forthcoming studies a quantitative PCR method e.g. Lightcycler (Roche) is preferable to enable the detection of minor differences in expression of keratin 1 and 10.

4.5 Capability of invasion
The influence of psoriasin on the capability of invasion was studied with a cell invasion assay. Normal human mammary epithelial cells (MCF10A) served as a control cell line while human mammary epithelial cancer cells (MB435) were used as test cells. MCF10AQ cells were compared with MCF10AHID cells while MB435 cells were compared to MB435 cells transfected with adenovirus carrying the HID5/psoriasin-gene. All of the cell types invaded the control membrane successfully which indicates that the assay was functional. The %
invasion was calculated for both MB435 and MCF10A according to the formula described in part 3.7 (data not shown). Cells expressing psoriasin did not show any difference in the capability of invasion compared to non-expressing cells in either of the two cell lines tested.
5. Discussion

The expression of psoriasin in conditions characterised by cellular and vascular hyperproliferation combined with abnormal differentiation, in particular the association with various forms of cancer, make psoriasin an interesting protein. The fact that cancer is common in the general population puts higher demands of the discovery of the pathways behind this complex group of diseases and the need for prognostic marker is therefore increasing. Although extensive efforts during the past decade the function of the protein psoriasin is yet unestablished. The purpose of this study was to investigate the expression pattern and the involvement of psoriasin in proliferation, sensitivity to apoptosis, differentiation process and capability of invasion in mammary epithelial cells and keratinocytes in vitro.

To identify the expression pattern of psoriasin several substances relevant for the pathology of psoriasis and the tumour environment were tested for their ability to induce the expression of psoriasin in vitro. The expression of psoriasin was successfully confirmed in keratinocytes in conditions where the cells loose attachment to the ECM. This condition had previously been described as a cause to the over-expression of psoriasin in mammary epithelial cells [Enerbäck et al 2002]. This study showed thereby that culturing cells in suspension (loss of attachment to ECM) up-regulates the psoriasin expression in keratinocytes as well as in mammary epithelial cells. The loss of attachment to ECM is characteristic for both psoriatic keratinocytes and cancer cells and this finding supports the hypothesis that the function of the protein is relevant for both of the two abnormal conditions.

When the over-expression had been established substances were tested for their ability to inhibit the psoriasin expression. Fascinating results were achieved when mammary epithelial cells were cultured in suspension and transfected with recombinant adenovirus carrying the Bcl2-gene. The inhibition was observed when doses of virus from 0.01μl up to 1μl were used. This might suggest that the inhibition of psoriasin is not dependent of the dose of Bcl2. The decreased expression due to the presence of Bcl2 is a very interesting discovery. The Bcl2-related protein family constitutes one of the most relevant classes of apoptosis-regulatory gene products [Kroemer 1997] among which Bcl2 is classified as an apoptotic inhibitor. Bcl2 is predominantly localised in the outer mitochondrial membrane but is additionally found in
the nuclear membrane and the ER [Reed 1994]. The protein suppresses apoptosis when targeted to the mitochondria. It neutralises proapoptotic proteins and has been reported to act by blocking p53-dependent apoptosis [Chiou et al 1994]. The function of Bcl2 is in accordance with the features of cellular hyperproliferation in both psoriasis and DCIS. These conditions are also characterised by high apoptotic rates which make this observation a very interesting discovery. Reactive oxygen species (ROS) are secreted from the mitochondria and this apoptotic inducer process is inhibited by Bcl2. Bcl2 acts by stabilising the mitochondrial membrane. It is therefore a possibility that ROS might induce the psoriasin expression. Another possibility could be that the apoptotic process per se induces the expression. Further experiments are needed to be able to answer these questions. At first the inhibition by Bcl2 might suggest counteracting roles for Bcl2 and psoriasin but one theory could be that the psoriasin and the Bcl2 proteins influence the same apoptotic mechanism and thus share function. If one of the apoptotic inhibition proteins already is present in the cell the expression of the other is not necessary. It is thereby possible that the inhibition of psoriasin by Bcl2 might be an indication of redundancy.

To find out whether the inhibition by Bcl2 is a general phenomenon, it is necessary to investigate the influence on other cell lines such as keratinocytes more thoroughly. So far the inhibition is observed in HEKn but not in HaCaT. One explanation could be that HaCaT is a relatively difficult cell line to transfect and a too low dose of virus has been used. HaCaT transfected with various doses of adenovirus carrying the Bcl2-gene are at present under investigation. An additional option is to perform the same experiment on a cell line which is constantly expressing psoriasin, e.g. MB468. MB468 have a natural up-regulation of psoriasin, i.e. the over-expression is not due to a viral promoter. It would therefore be interesting to see if Bcl2 is capable of inhibiting the naturally occurring expression. The relationship between Bcl2 and psoriasin is thus subject for further research and exciting experiments are in prospects.

The involvement of psoriasin in the proliferation process were investigated in two different human mammary epithelial cancer cell lines and one transformed mouse cell line. Differences in the proliferation rate between psoriasin-expressing and non-expressing cells were analysed. The results were visually interpreted and so far no influence of psoriasin has been observed. An option for forthcoming studies would be to count the number of the cells to achieve a
more accurate comparison. Any minor differences in proliferation rate would then be detected.

In the second functional approach psoriasin-expressing and non-expressing cells were exposed to apoptotic environments. The cells were exposed to loss of attachment to ECM, malnutrition, over-expression of p53 and presence of ROS to test their sensitivity to apoptosis. So far no differences in the sensitivity have been observed. The experiment will in the future be performed on HEK\textsubscript{n} which is a normal keratinocyte cell line.

In order to investigate the involvement of psoriasin on the differentiation process RT-PCR was performed on differentiating and non-differentiating keratinocytes. Psoriasin-expressing and non-expressing keratinocytes were exposed to high extra cellular calcium concentration (4mM), 1µM of wortmannin or culturing in suspension using polyH\textsubscript{EM}E-coated dishes to induce the differentiation process. The markers for differentiation were represented by keratin 1 and 10. At present this investigation is incomplete since priority has not been placed on this approach. The approach is very interesting and the project is about to start using a quantitative PCR method, e.g. Lightcycler (Roche). The Lightcycler enables detection of minor differences in the expression of keratin 1 and 10.

In the fourth functional approach the influence of psoriasin on the capability of invasion was examined. A cell invasion assay using BD BioCoat\textsuperscript{TM} Matrigel\textsuperscript{TM} Invasion Chamber was used. The assay includes two types of membrane inserts which the cells are allowed to invade. The control membrane consists of a porous polyethylene teraphthalate (PET) membrane that allows the cells to pass through easily. The Matrigel-coated PET-membrane is a functional \textit{in vitro} extracellular matrix (ECM) system and enables only cells with an invasive potential to enter the system. In the assay cells are invading the membranes towards a chemoattractant, in this case serum containing media. A large number of cells invading the control membranes verify the proper function of the chemoattractant and the assay itself. The cell lines used in this assay were MB435 and MCF10A which served as a control cell line. MB435 has been reported as a cancer cell line with a moderate invasive potential \textit{[personal communication, Dr Kornelia Polyak at Dana-Farber Cancer Institute in Boston]} and was therefore thought to be a suitable test cell line since changes, both increases and decreases, in invasion can be detected. During the experiment the number of MB435 cells which invaded the Matrigel-membranes was low. As a cancer cell line the expected result would be a higher capability of invasion for
MB435. The experiment was repeated twice with the same cell line and the results were identical. Comparing the psoriasin-expressing and non-expressing cells implies no influence of psoriasin in the capability of invasion. This result is however not consistent with the recently published data where psoriasin-expressing cells are slightly more invasive [Emberely et al 2003]. That particular study was on the other hand performed on MB231 which is considered to be equal to MB435. A cell line is however always a cell line and certain observations could therefore be representative for that cell line only. Since the psoriasin expression is markedly diverse between in situ carcinomas and invasive tumours it is possible that the function of the protein might be associated with the capability of invasion. This approach is thus appealing and for future studies it is desirable to perform the invasion approach repeatedly and simultaneously with MB435 and MB231 comparing non-transfected and transfected cells with adenovirus carrying HID5/psoriasin.

All of the functional assays used in this study are interesting approaches towards the discovery of the function for psoriasin. A lot of exciting experiments are in progress and hopefully the function of the protein psoriasin will in a near future be established.
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7. References


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8. Appendix

RNeasy Mini Kit Protocol for isolation of total RNA from animal cells (Qiagen)

The amount of starting material is limited to an absolute maximum of $1 \times 10^7$ cells. A confluent Ø100 mm cell culture plate holds $0.7 \times 10^7$ cells hence the number of cells never exceeds the limit.

1. Trypsinise the cells and centrifuge them in a 50ml centrifuge tube at 2500 rpm for 3 minutes. Discard the supernatant and transfer the cells to an RNase-free Eppendorf tube. Centrifuge the cells at 2500 rpm for 3 minutes and completely aspirate the supernatant.

2. Add 300µl of RLT-buffer containing β-mercaptoethanol and homogenise the sample with a homogeniser. Add additionally 300µl of RLT Buffer containing β-mercaptoethanol.

3. Add one volume (600µl) of 70% room temperature ethanol and mix well by pipetting.

4. Apply up to 700µl of the sample, including any precipitate that may have formed to an RNeasy mini column placed in a 2ml collection tube. Centrifuge for 30 seconds at 12000 rpm and discard the flow-through. Load any aliquots successively onto the RNeasy column and centrifuge as above. Discard the flow-through.

5. Add 700µl of Buffer RW1 to the RNeasy column. Centrifuge for 30 seconds at 12000 rpm to wash the column. Discard the flow-through and the collection tube.

6. Transfer the RNeasy column into a new 2ml collection tube and pipet 500µl of Buffer RPE onto the column. Ensure that ethanol is added to Buffer RPE before use. Centrifuge for 30 seconds at 12000 rpm to wash the column. Discard the flow-through.

7. Add another 500µl Buffer RPE to the column. Centrifuge for 2 minutes at 12000 rpm to dry the RNeasy silica-gel membrane.

8. Transfer the RNeasy column to a new 1.5ml collection tube. Pipet 30µl of RNase-free water directly onto the RNeasy silica membrane. Centrifuge for 1 minute at 12000 rpm to elute the RNA. Store in -70°C.