PH.D. THESIS

Investigation of xenobiotic transporters in human keratinocytes and in human skin

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Introduction

The human body comes into contact with numerous unwanted and harmful components from the environment, which is inevitable during processes requiring environmental connection, such as digestion and respiration. Substances that are foreign to an organism are called xenobiotics, these molecules differ greatly in their size and in their chemical characteristics. There is a multi-leveled defensive system in order to prevent xenobiotics from entering the body, to cope with all the compounds already absorbed and to dispose them. Xenobiotic transporters from the ABC transporter protein superfamily play a very important role in this protective machinery.

Ten members of the ABC transporters are known to play a considerable role in multidrug resistance of cancer cells, these proteins are the so-called xenobiotic transporters. Their common features include extremely broad substrate specificity, which has serious consequences in chemotherapy: the presence of xenobiotic transporters in tumors increases the resistance of cancer cells against chemotherapeutics. Earlier research focused on the roles of these transporters in chemotherapy resistance, but recently interesting data have emerged regarding their physiological functions. In general, they are involved in normal tissue protection against environmental toxins and against endogenous compounds produced in cells during normal metabolic processes or in response to stress.

The epidermis is the largest and most important mechanical and chemical permeability barrier in the body. It has been suggested that xenobiotic metabolizing enzymes such as cytochrome P450 proteins and xenobiotic transporters contribute to the biochemical barrier functions of the epidermis.

The expression of several xenobiotic transporters has been detected in normal human keratinocytes and the membrane associated presence of ABCB1 and ABCC1 has also been described in human epidermis. Studies on murine skin showed that xenobiotic transporters could affect drug absorption through skin, their distribution and availability in the body.

Aims

- There is only limited information in the literature about the function of xenobiotic transporters in human skin. Our aim was to provide detailed transcriptional data on ABCB1 (Pgp/MDR1), ABCC1-6 (MRP1-6) and ABCG2 (BCRP) genes during proliferation and differentiation of human keratinocytes.
- Our results on the transcriptional pattern of ABCC4 and ABCG2 genes suggested their proliferation-associated expression. Therefore
    - we aimed to investigate the expression of ABCC4 and ABCG2 proteins in detail in our *in vitro* keratinocyte model systems
    - and we decided to examine their role in keratinocyte proliferation using siRNA-mediated gene specific silencing and chemical inhibition.
- Based on our *in vitro* results we extended our study on ABCC4 and ABCG2 transporters to human skin, with particular attention to therapeutic considerations.
    - We examined the expression of the ABCC4 transporter protein in the skin of psoriatic patients. We also aimed to investigate the effect of transporter inhibitors and methotrexate – a drug used in the therapy of psoriasis – on cultured keratinocytes.
    - We studied the expression of the ABCG2 transporter in non-melanoma skin cancers. Our aim was to elucidate the role of ABCG2 as a potential target molecule through which the efficacy of PDT can be enhanced.

Methods

- Keratinocyte proliferation/differentiation models using HaCaT cells and normal human keratinocyte cultures
- Real-time RT-PCR
- Western blot and immunocytochemistry were used to determine ABCC4 and ABCG2 protein expression
- Flow cytometric detection of plasma membrane ABCG2 and intracellular porphyrin levels
- siRNA-mediated silencing of ABCC4 and ABCG2 genes
- 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for measuring cell viability
- Real-time cell proliferation analysis (XCELLigence)
- Immunohistochemical staining to determine ABCC4 and ABCG2 expression in tissue samples
- *In vitro* photodynamic therapy using HaCaT cells

Results

1. The ABCC4 and ABCG2 transporters are expressed in a proliferation-related manner in *in vitro* models of keratinocyte proliferation and differentiation

We investigated the expression profile of ABCB1, ABCC1-6 and ABCG2 genes during keratinocyte proliferation and differentiation by real-time RT-PCR. Of the examined xenobiotic transporter genes, ABCC4 and ABCG2 exhibited a characteristic proliferation-related expression pattern in both *in vitro* models, in synchronized HaCaT cells and in differentiating NHKs. The mRNA levels of ABCB1, ABCC1, ABCC3, ABCC5 and ABCC6 transporters showed no or only insignificant changes, the ABCC2 gene was induced only in synchronized HaCaT cells.

The model of synchronized HaCaT cells is based on a two-week procedure of contact inhibition and serum starving, where the cells become highly differentiated and cease to proliferate. After passaging and re-adding the serum to the medium the cells exit simultaneously from this quiescent state. In parallel with this, the expression of differentiation markers decreases, whereas proliferation markers are downregulated. In

synchronized HaCaT cells, the ABCC4 and ABCG2 mRNA levels were induced during the intensive proliferation of the cells.

Terminal differentiation of normal human keratinocytes was induced by means of contact inhibition and increased Ca^{2+} concentration. We detected a continuous decrease in the expression of ABCC4 and ABCG2 genes during the differentiation of the cells. According to our Western blot and immunocytochemistry studies ABCC4 and ABCG2 proteins were present at high levels in proliferating cells in both models, showing a good correlation with our gene expression data.

2. Inhibition of ABCC-type transporters abrogated the proliferation of normal human keratinocytes

HaCaT cells and normal human keratinocytes were treated with the ABCC-type transporter inhibitor probenecid and the ABCG2 specific inhibitor molecule, Ko-134. The proliferation of the cells was monitored real-time during the 72 hour treatment. HaCaT cells treated with probenecid or Ko-134 showed a proliferation rate similar to control cells. Specific silencing of ABCC4 and ABCG2 genes did not affect the viability of HaCaT cells. The disruption of ABCC-type transporter function in normal human keratinocytes did not result in cell death, however cell proliferation was inhibited by probenecid treatment. Incubation with Ko-134 had no effect on the proliferation and viability of normal human keratinocytes. We concluded that ABCC-type xenobiotic transporters, including ABCC4, could play a significant role in the proliferation of normal human keratinocytes.

3. The ABCC4 transporter is overexpressed in hiperproliferating keratinocytes in psoriatic lesions

Epidermal dendritic cells showed ABCC4 immunopositivity in healthy skin and in psoriatic non-lesional skin. However, in psoriatic lesional epidermis basal layer keratinocytes expressed the ABCC4 protein at high levels. Our results suggested, that the ABCC4 transporter may contribute to abnormal keratinocyte proliferation observed in psoriasis. The protein could also modify the efficacy of drugs used in the therapy of psoriasis, particularly that of methotrexate.

4. ABCC-type transporters play an important role in the resistance of HaCaT cells to methotrexate treatment

Methotrexate is an antifolate commonly used in the therapy of psoriasis and a known substrate of several xenobiotic transporters. HaCaT cells showed significant resistance to short-term methotrexate treatment. This phenomenon may be caused by xenobiotic transporters expressed in the cells. We applied xenobiotic transporter inhibitors during the four-hour methotrexate treatment in order to examine, which transporter protein is responsible for methotrexate resistance. The ABCC-type transporter inhibitors probenecid and indomethacin significantly increased the toxic effect of methotrexate on HaCaT cells. A recently characterized ABCC4 specific inhibitor, 4-(2-aminoethyl)-benzenesulfonyl-fluoride exerted a toxic effect on cells, thus this molecule is not suitable for further experiments on living cells as a transporter inhibitor.

5. The ABCG2 transporter is expressed at high levels in psoriatic lesions and in several types of non-melanoma skin cancers

A weak staining of the ABCG2 transporter protein was detected in the basal layer keratinocytes in healthy skin, while the suprabasal epidermal layers were negative. Non-lesional skin of psoriatic patients showed a staining pattern of ABCG2 similar to the healthy samples. However, in psoriatic lesional epidermis high levels of ABCG2 protein was detected in the suprabasal layers. The hyperproliferating basal layer keratinocytes showed only very low ABCG2 immunopositivity. These results are in accordance with our previous *in vitro* data, suggesting that the ABCG2 transporter does not play a significant role in keratinocyte proliferation. We observed ABCG2 protein overexpression in non-melanoma skin cancers. ABCG2 positivity was not characteristic to tumor cells in the case of basal cell carcinomas, however, suprabasal layers of the epidermis near the lesion showed high level ABCG2 transporter expression. In squamous cell carcinoma, Bowen carcinoma, keratoacanthoma and epidermal hyperplasia samples the cancerous cells revealed significant ABCG2 positivity.

6. The accumulation of free intracellular porphyrins depends on the expression of ABCG2 in HaCaT cells

We detected a high-level of ABCG2expression in the epidermis of non-melanoma skin cancer samples, such as basal cell carcinoma and squamous cell

carcinoma. Photodynamic therapy is increasingly used as a successful treatment option in these skin conditions. It is known that the ABCG2 protein plays an important role in removing excess free porphyrins from the cells, therefore we hypothesized that ABCG2 may be a target molecule to improve the efficacy of photodynamic therapy. We characterized an *in vitro* photodynamic therapy model using HaCaT cells. The cells were treated with delta-aminolevulinic acid (DALA), which results in a significant increase in intracellular free porphyrin levels. Intracellular porphyrin content of HaCaT cells after a four-hour DALA pretreatment was determined by flow cytometry. We investigated whether the porphyrin-extruding ability of HaCaT keratinocytes depends on the proliferation/differentiation state of the cells. Our previous experiments revealed that the ABCG2 protein was nearly undetectable in highly differentiated keratinocytes, while high levels of this transporter protein were present in proliferating cells. We next examined the correlation between the proliferation/differentiation state of HaCaT keratinocytes and porphyrin accumulation. Highly differentiated HaCaT cells accumulated ~ 7.5 times more porphyrin than did proliferating cells. Moreover, inhibition of the ABCG2 function with Ko-134 during DALA treatment did not result in a further increase in porphyrin level in differentiated keratinocytes. The highly proliferating cells characterized by elevated levels of the ABCG2 protein displayed a significant increase in free porphyrin upon ABCG2 inhibition by Ko-134 during DALA pretreatment.

7. Specific inhibition of ABCG2 transporter function increases the efficacy of photodynamic treatment in HaCaT cells

In order to examine the effect of the specific inhibition of ABCG2 function on porphyrin-mediated photosensitivity we characterized an *in vitro* model of photodynamic therapy using HaCaT cells. Cells were sensitized by a four-hour DALA pretreatment and then irradiated with an Aktilite photodynamic therapy light source. DALA pretreatment in combination with Ko-134 significantly and dose-dependently increased the sensitivity of HaCaT keratinocytes to irradiation with therapeutic light.

Summary

- According to our *in vitro* studies, ABCC4 and ABCG2 transporters showed a proliferation-associated expression pattern in keratinocytes.
- The ABCC4 transporter may have a regulatory role in keratinocyte proliferation; the protein is overexpressed in the hyperproliferating keratinocytes in psoriatic lesional skin.
- ABCC-type xenobiotic transporters – including ABCC4 – contribute to methotrexate resistance of HaCaT cells.
- ABCG2 was highly expressed in non-melanoma skin cancers.
- The ABCG2 protein removes the photosensitizer porphyrin molecules from the cells, and may well be a target molecule to enhance the efficacy of photodynamic therapy.

Publications related to the Ph.D. thesis:

Bebes A, Kis K, Nagy T, Kurunczi A, Polyánka H, Bata-Csörgő Zs, Kemény L, Dobozy A, Széll M: **The expressions of ABCC4 and ABCG2 xenobiotic transporters in human keratinocytes are proliferation-related.** *Arch Dermatol Res* (2011) in press, doi:10.1007/s00403-011-1174-4, IF: 2,011

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