RNAi-mediated silencing of matrix metalloproteinase 1 in epidermal keratinocytes influences the biological effects of interleukin 17A

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Matrix metalloproteinases (MMPs) are important for the pathogenesis of psoriasis and other autoimmune disorders. In the extracellular matrix, accumulation of proinflammatory cytokines, such as interleukin 17A (IL-17A), leads to induction of several MMPs, including MMP1. MMPs change the composition and other properties of the extracellular matrix. These changes facilitate tissue remodeling and promote the development of psoriatic plaques. The aim of this study was to explore how MMP1 silencing might influence the biological effects of IL-17A on migration and proliferation of human epidermal keratinocytes and the expression of genes involved in their division and differentiation. The experiments were performed with MMP1-deficient and control epidermal keratinocytes, HaCaT-MMP1 and HaCaT-KTR, respectively. Cell proliferation and migration were assessed by comparative analysis of the growth curves and scratch assay, respectively. To quantify cell migration, representative areas of cell cultures were photographed at the indicated time points and compared to each other. Changes in gene expression were analyzed by real-time PCR. The obtained results demonstrated that MMP1 silencing in the cells treated with IL-17A resulted in downregulation of MMP9 and -12, FOSL1, CCNA2, IVL, KRT14 and -17 as well as upregulation of MMP2, CCND1 and LOR. Moreover, MMP1 silencing led to a decrease in cell proliferation and an impairment of cell migration. Thus, MMP1-deficiency in epidermal keratinocytes can be beneficial for psoriasis patients that experience an accumulation of IL-17 in lesional skin. Knocking down MMP1 could influence migration and proliferation of epidermal keratinocytes in vivo, as well as help to control the expression of MMP1, -2, -9 and -12, CCNA2, CCND1, KRT14 and -17 that are crucial for the pathogenesis of psoriasis.

Key words: matrix metalloproteinase 1; psoriasis; interleukin 17; small hairpin RNA; gene silencing.
Plaque psoriasis (psoriasis vulgaris) is one of the most abundant chronic non-infectious skin conditions (Greb et al., 2016). According to the World Health Organization, the prevalence of psoriasis worldwide is ~3% (Michalek et al., 2017). In mainland Russia, the prevalence of psoriasis is ~1.9% (Khamaganova et al., 2015). The most distinctive hallmark of psoriasis is the appearance of thick scaly plaques on the patient’s skin. In 21.1% and 5.7% of cases, respectively, the disease targets nails and joints (Rukavishnikova, 2009; Mishina et al., 2013). Moreover, psoriasis is often accompanied by comorbidities, such as cardiovascular disease, type II diabetes and atherosclerosis (Batyrshina, Sadykova, 2014).

At the molecular level, the development of psoriatic plaques leads to differential expression of thousands of genes (Zolotarenko et al., 2016), including the genes involved in proliferation and the terminal differentiation of epidermal keratinocytes. In lesional skin, KRT1, KRT5 and KRT10 are downregulated (Rao et al., 1996; Jin, Wang, 2014), whereas KRT14, KRT16, and KRT17 are upregulated (Al Robaee, 2010; Jin, Wang, 2014). The late differentiation markers loricrin (LOR) and filaggrin (FLG) are expressed at a lower level. In contrast, the expression level of the early differentiation marker involucrin (IVL) is higher (Soboleva et al., 2014a). The proliferation marker MKI67 (Yazici et al., 2005) and cyclin A2 (Manczinger, Kemény, 2013) are upregulated, while cyclin D1 is downregulated (Reischl et al., 2007).

The proinflammatory cytokine interleukin 17A (IL-17A) plays a key role in the pathogenesis of psoriasis. Specifically, IL-17A contributes to the inflammatory response by inducing the chemokines CCL20, CXCL1, CXCL2, and CXCL8. Their secretion to the extracellular matrix promotes the migration of activated immune cells to lesional epidermis. In turn, the accumulation of immune cells in lesional skin stabilizes there the inflammatory process (Seo et al., 2017).

This concept fits well with emerging results from clinical trials with IL-17A-specific antibodies (Canavan et al., 2016). According to the clinical data, ~85–90% of patients with moderate and severe psoriasis achieve PASI75 after completion of the therapy. Moreover, the tested IL-17A-specific antibodies exhibit low immunogenicity. On the other hand, these medicines affect the patients’ ability to respond to pathogens. In addition, people who experience allergic reactions or were advised from taking injections of IL-17A-specific antibodies.

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Materials and methods

Cell culturing. The experiments were performed with MMP1-deficient and control immortalized epidermal keratinocytes, HaCaT-MMP1 and HaCaT-KTR, respectively. The mentioned cell lines were obtained as described previously (Mogulevtseva, Mezentsev, 2017). The cells were cultured in DMEM medium, supplemented with 5% embryonic calf serum, L-glutamine (PanEco, Russia) and antibiotic-antimycotic (ThermoFisher Scientific) Kit (ThermoFisher Scientific) was used to quantify RNA according to the manufacturer’s protocol.

Preparation of total RNA. The total RNA was purified with TRIZOL (ThermoFisher Scientific) as described previously (Chomczynski, Mackey, 1995). Purity and integrity of the obtained RNA were verified using non-denaturing 1.5% agarose gel electrophoresis. A Qubit RNA BR Assay Kit (ThermoFisher Scientific) was used to quantify RNA according to the manufacturer’s protocol.

Real-time PCR. Before the experiment, RNA was converted to cDNA using an MMLV RT kit (Evrogen, Russia). The experiments were carried out in the Eco real-time PCR system (Illumina, USA) according to the instructions supplied by the manufacturer. The primers used in this study were taken from the NCBI Probe database (NCBI Probe, 2015). The ACTB assay was used as an endogenous control. The results were analyzed using preinstalled software supplied by the manufacturer. Each probe was run in triplicates. Overall, three independent experiments were performed.

Proliferation assay. To assess the cell proliferation rate, cells were plated at 40,000 cells/well in 6-well plates. To obtain cell suspensions, randomly chosen samples were treated with 0.25% trypsin-EDTA (PanEco) on a daily basis. The cells...
suspensions were stained with 0.2% trypan blue and counted with a hemocytometer. Then, the cell counts were plotted against the incubation time to generate the growth curves in linear coordinates. To assess changes in cell proliferation rates, the data were represented in semilogarithmic coordinates and subjected to linear regression analysis. Slopes of these lines served us as the estimates of the cell proliferation rates. Overall, three independent experiments were performed.

**Scratch assay.** To assess cell migration, the designated cell lines were cultured until confluence. The cell monolayer was scratched with a pipette tip to form a 1.25 mm-wide cell-free area across the center of the well. Then, the remaining cells were cultured for 5–6 days and the most representative cell-free areas were photographed on a daily basis. These areas were quantified using the “Freehand selection” tool of “ImageJ” freeware (Schindelin et al., 2015).

**Statistical analysis.** The data obtained were represented as the mean ± standard error (m ± SE). The statistical differences between the means were assessed by a one-way analysis of variances. If p values were less than 0.05, the means were considered to be significantly different.

**Results**

**Influence of MMP1 silencing on gene expression**

Culturing HaCaT-KTR and HaCaT-MMP1 cells in the presence of IL-17A resulted in a differential expression of matrix metalloproteinases, cytokeratins, proliferation markers and terminal differentiation markers of epidermal keratinocytes (Fig. 1).

Particularly, the exposure of HaCaT-KTR cells to IL-17A led to upregulation of MMP9 and MMP12 (11.15 ± 1.67 and 7.58 ± 1.14, respectively) and downregulation of MMP2 (0.37 ± 0.05). In the same time, their expression levels in HaCaT-MMP1 cells were 2.29 ± 0.34, 2.44 ± 0.37 and 0.98 ± 0.15, respectively (Fig. 1, a). Moreover, MMP1 expression in MMP1-deficient cells remained relatively low (0.28 ± 0.04), despite the cells were exposed to a high concentration of IL-17A.

The expression of the cell proliferation marker MKI67 was increased in both cell lines (3.80 ± 0.57 in HaCaT-KTR cells and 5.66 ± 0.85 in HaCaT-MMP1 cells, p = 0.36). In contrast, cyclins CCNA2 and CCND1 were downregulated (Fig. 1, b). Particularly, CCNA2 expression levels were 0.32 ± 0.05 in HaCaT-KTR cells and 0.13 ± 0.02 in HaCaT-MMP1 cells, while CCND1 expression levels were 0.13 ± 0.02 and 0.71 ± 0.11, respectively.

Moreover, we observed slight changes in the expression levels of the terminal differentiation markers in HaCaT-KTR cells. Particularly, the expression levels of IVL, LOR and FLG were 1.44 ± 0.13, 1.15 ± 0.11 and 1.26 ± 0.27, respectively (Fig. 1, c), whereas the expression levels of the mentioned genes in HaCaT-MMP1 cells were 1.01 ± 0.11 (IVL), 1.79 ± 0.17 (LOR) and 1.89 ± 0.14 (FLG). In HaCaT-KTR cells, the expression levels of cytokeratins did not exceed 1.5 compared to the negative control, except KRT14 and KRT17, which were upregulated (1.86 ± 0.14...
and 1.64 ± 1.15, respectively). In contrast, KRT17 was significantly downregulated in HaCaT-MMP1 (0.19 ± 0.02) compared to HaCaT-KTR cells.

**Influence of MMP1 silencing on cell proliferation**

Comparative analysis of the growth curves revealed that HaCaT-MMP1 and HaCaT-KTR cells remained in the active growth phase for the time of the experiment (Fig. 2). The time-dependences of cell growth in linear coordinates (Fig. 2, a) suggested that the cells grew monotonously, i.e. for the time of observation, the growth curves did not reach saturation. At the same time, a semilog transformation of the growth curves (Fig. 2, b) revealed a high correlation between time and cell numbers ($r > 0.99$; $p < 0.001$). Furthermore, MMP1 silencing caused a delay in cell growth (Fig. 2, a). According to the results of linear regression, the proliferation rates of HaCaT-KTR and HaCaT-MMP1 cells were 0.0090 ± 0.0003 and 0.0078 ± 0.0003, respectively.

**Influence of MMP1 silencing on cell migration**

Analysis of cell migration showed that the motility of HaCaT-MMP1 was significantly impaired compared to HaCaT-KTR cells (Fig. 3, a). In contrast, exposure of both cell lines to IL-17A stimulated cell migration. In this respect, MMP1-deficiency resulted in an 11.48-fold decrease of the migration constant, whereas exposure of HaCaT-KTR and HaCaT-MMP1 cells to IL-17A caused 1.43- and 3.63-fold increases of the corresponding migration constants (Fig. 3, b).

**Discussion**

IL-17A is one of proinflammatory cytokines that are implicated in the pathogenesis of various autoimmune diseases including psoriasis (Korotaeva, 2016). In psoriasis, IL-17A is accumulated in lesional skin. After secretion by T-helper 17 (Th17) immune cells into the extracellular matrix, IL-17A activates the IL-17RA receptor, which is located in the plasma membrane of immunocytes, such as macrophages, and resident skin cells primarily keratinocytes, dendritic cells and fibroblasts. The interaction of IL-17A with IL-17RA results in the induction of various proinflammatory cytokines (TNF, IL-1β, and IL-6, etc.) and chemokines (IL-8, CXCL1, CXCL2 and CCL20) (Mills, 2008; Seo et al., 2017). The induced cytokines influence proliferation and differentiation of epidermal keratinocytes, whereas chemokines promote the
migration of immune cells, such as neutrophils and monocytes to the skin. The former causes the development of psoriatic plaques. The latter stabilizes the inflammatory response in psoriatic plaques.

The concentration of IL-17A in the blood serum of healthy volunteers usually does not exceed 10 pg/mL (Shilova et al., 2015). In contrast, the biological effects of IL-17A in cultured epidermal keratinocytes and fibroblasts, such as activation of protein kinases (Peric et al., 2008, 2009), induction of proinflammatory cytokines (Tohyama et al., 2009; Cho et al., 2012) or influencing cell proliferation (Ma, Jia, 2016) require an incubation of cells in the presence of 10–100 ng/mL IL-17A. Presumably, this difference can be explained by the fact that in psoriasis, T\(_{\text{yp}}\) cells, which are the main source of IL-17A in the body, are predominantly located in the inflamed tissue where they are needed to stabilize the inflammatory response. For this reason, we treated epidermal keratinocytes with 50 ng/mL IL-17A (Starodubtseva et al., 2011).

Culturing HaCaT-KTR and HaCaT-MMP1 cells in the presence of IL-17A, we anticipated that the genes involved in the terminal differentiation and proliferation of epidermal keratinocytes would be differentially expressed. Primarily, we expected to see changes in gene expression similar to ones that occur during the development of psoriatic plaques. In this respect, changes in the expression of IVL, MK167, KRT14 and KRT17 that we observed in HaCaT-KTR cells, i.e. the cell line that expressed scrambled shRNA, did not surprise us (see Fig. 1). We also wanted to explore how MMP1 silencing could influence the expression of genes that were implicated in the pathogenesis of psoriasis. Notably, upregulation of MK167, MMP9 and MMP12 as well as downregulation of CCNA2 and CCND1 occur in both cell lines, i.e. it is unlikely that they are caused by MMP1-silencing (see Fig. 1, a and b). In the same time, lower expression levels of MMP9 and MMP12 as well as a higher expression level of MMP2 in HaCaT-MMP1 suggest that the expression of MMP1-specific shRNA could help to control MMPs expression in lesional psoriatic skin (Nair et al., 2009). Moreover, changes in the expression of the terminal differentiation markers, such as downregulation of IVL (\(p = 0.05\)) and upregulation of LOR (\(p = 0.03\)) in HaCaT-MMP1 cells (see Fig. 1, c) are also opposite to ones that occur in lesional psoriatic skin. However, we did not discover significant differences in FLG expression between HaCaT-MMP1 and HaCaT-KTR (\(p = 0.29\)).

Notably, exposure of both cell lines to IL-17A does not cause significant changes in the expression of many cytokeratins (see Fig. 1, d). This can be explained by the fact that HaCaT cells, i.e. the cell line that was used to generate HaCaT-KTR and HaCaT-MMP1 cells, and primary epidermal keratinocytes react differentially to treatment with IL-17A. Particularly, culturing the primary cells in the presence of 50 ng/mL IL-17A results in downregulation of KRT10, FLG, LOR and IVL (Noh et al., 2010), whereas treatment of HaCaT with the same concentration of IL-17A does not cause any significant changes in the expression of the named genes (Seo et al., 2012). In contrast, we report here that the incubation of HaCaT-KTR cells with IL-17A results in upregulation of KRT14 and KRT17. These changes in gene expression are similar to ones that occur in lesional psoriatic skin (Nair et al., 2009). However, it is even more important that MMP1 silencing downregulates both genes. For instance, it results in more than a 10-fold downregulation of KRT17. In the published papers, KRT17 is often referred to as a “key gene” of psoriasis (Al Robaee, 2010; Jin, Wang, 2014). Suppression of KRT17 in the skin of lab animals prevents hyperplasia, i.e. thickening of the epidermis due to more intensive cell division and lowering the intensity of the inflammatory process. In contrast, induction of KRT17 stimulates the secretion of T\(_{\text{p}}\) chemokines, such as CXCL5, CXCL9, CXCL10 and CXCL11 (Al Robaee, 2010). In this respect, we assume that MMP1 silencing could be beneficial for psoriasis patients to attenuate the inflammatory response and suppress hyperplasia in lesional psoriatic skin.

Comparative analysis of gene expression in HaCaT-MMP1 and HaCaT-KTR cells treated with IL-17A reveals that HaCaT-MMP1 cells express less CCNA2 and more CCND1 (see Fig. 1, b). However, differences in the expression of the proliferation marker MK167 between these two cell lines are insignificant (\(p = 0.36\)). According to the previously published data, CCND1 is required for the transition from G\(_2\)-phase of the cell cycle to S-phase, whereas CCNA2 is needed for the transition from G\(_1\)-phase to M-phase (Matsushime et al., 1992; Pagano et al., 1992). Furthermore, the changes in the expression of CCND1 and CCNA2 that the others observe in psoriatic lesional skin are opposite to the ones we see in MMP1-deficient cells. Particularly, comparative analysis of skin samples obtained from lesional and uninvolved skin reveals a two-fold decrease for CCND1 (Reischl et al., 2007) and an 8.7-fold increase for CCNA2 (Maneczinger, Kemény, 2013) in lesional skin. This shift in the cytokine balance is also in line with the results of our cell proliferation assay (see Fig. 2). According to the obtained data, HaCaT-MMP1 cells exhibit a 1.15-fold decrease in the constant of proliferation compared to HaCaT-KTR cells. At the same time, IL-17A does not stimulate proliferation of HaCaT cells in vitro (Soboleva et al., 2014b). Hence, we propose that MMP1 silencing could also exert an antiproliferative effect in lesional psoriatic skin that accumulates IL-17A.

Moreover, culturing HaCaT-KTR cells in the presence of IL-17A results in differential expression of the matrix metalloproteinases (see Fig. 1, a) MMP2 (0.37 ± 0.05), MMP9 (11.15 ± 1.67) and MMP12 (7.58 ± 1.14). To the reference, similar changes in the expression of the named metalloproteinases occur in lesional psoriatic skin. For instance, one of the previous studies performed in our lab revealed that in lesional psoriatic skin, MMP2 was downregulated (0.77 ± 0.23), whereas MMP9 and MMP12 were upregulated (4.2±0.65-fold and 17.25±5.80-fold, respectively) compared to uninvolved skin (Starodubtseva et al., 2011). In this study, we show that MMP2, MMP9 and MMP12 expression levels in MMP1-deficient cells (0.98 ± 0.15, 2.29 ± 0.34 and 2.44 ± 0.37, respectively) are comparable to the negative control, i.e. HaCaT-KTR, untreated with IL-17A. In this respect, the obtained results suggest that MMP1 silencing could be used in vivo to control the expression of the mentioned matrix metalloproteinases, primarily MMP9 and MMP12.

In this paper, we also report that IL-17A stimulates migration of both HaCaT-KTR and HaCaT-MMP1 cells (see Fig. 3). According to the published data, IL-17A promotes the
MMP1 silencing in epidermal keratinocytes influences the biological effects of IL17A

J.A. Mogulevtseva
A.V. Mezentsev, S.A. Bruskin

The following molecules participating in MMP1-induced activation of PAR1 receptor.

In turn, we report that culturing the cells in the presence of either MMP9 or TNF significantly slows the migration down.

Moreover, treatment of the cells with antibodies specific to TNF induces cell migration in a dose-dependent manner. The authors of the cited paper report that TNF induces cell migration in a dose-dependent manner. Moreover, treatment of the cells with antibodies specific to either MMP9 or TNF significantly slows the migration down. In turn, we report that culturing the cells in the presence of IL-17A induces MMP9 and MMP12. According to our data, HaCaT-KTR cells where MMP9 is induced gain a higher motility, compared to HaCaT-MMP1 cells where MMP9 is downregulated (see Fig. 1, a).

In conclusion, we would like to acknowledge that the results of this study suggest reconsidering the traditional role of MMP1 as a proteolytic enzyme. Obviously, the influence of MMP1 silencing on the expression of cyclins and cytokeratins as well as the negative effect on cells proliferation can be explained by the direct participation of this enzyme in intracellular signalling pathways. First, this is possible due to some products of the MMP1-catalyzed reaction known as matrixins mediate the signalling mechanisms (Wells et al., 2015). Secondly, some matrix metalloproteinases, such as MMP1, directly interact with PAR receptors (Boire et al., 2004). Following the binding to the G-protein coupled PAR1 receptor (Fig. 4), MMP1 cuts N-terminal peptide. Then, this peptide binds to the receptor as a ligand and they form an active ligand-receptor complex. On the cytoplasmic side of the cell membrane, the complex interacts with one of G-proteins (Gα12/13, Gαi, Gq, Gβγ). Each of these proteines activates a unique pattern of signalling pathways that may result in a different outcome for the cell. Although the factors that influence the interaction of PAR receptors with particular G-proteins still need to be studied, activation of the same receptor in different physiological conditions may cause a shape change, changes in cell behavior, induction of certain genes or activation of protein secretion. Importantly, similar changes are also observed in the pathogenesis of psoriasis. As we believe, the key findings of this paper open an opportunity for the creation of a new therapeutic approach that can be used for psoriasis. In this respect, we would like to mention that new recombinant viruses were proposed to deliver the desired genes to the diseased organs and tissues and several therapeutic approaches were proposed to treat human genetic disorders (Hacein-Bey-Abina et al., 2002; Bainbridge et al., 2008). Moreover, viral transfection was successfully used to treat psoriasis in humanized animals (Jakobsen et al., 2009).

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Conflict of interest

The authors declare no conflict of interest.

References


Vegfors J., Ekman A.K., Stoll S.W., Bivik Eding C., Enerbäck C. 
Pсориасин (S100A7) promotes stress-induced angiogenesis. Br. J. 
Wu Y., Zhu L., Liu L., Zhang J., Peng B. Interleukin-17A stimulates migration of periodontal ligament fibroblasts via p38 
Yazici A.C., Tursen U., Apa D.D., Ikizoglu G., Api H., Baz K., Tas- 
delen B. The changes in expression of ICAM-3, Ki-67, PCNA, 
and CD31 in psoriatic lesions before and after methotrexate treat- 
s00403-005-0602-8. 
Zolotarenko A., Chekalin E., Mesentsev A., Kiseleva L., Gribanova 
E., Mehta R., Baranova A., Tatarinova T.V., Piruzian E.S., 
Bruskin S. Integrated computational approach to the analysis of 
RNA-seq data reveals new transcriptional regulators of psoriasis. 