Original paper

Differences in expression of genes related to drug resistance and miRNAs regulating their expression in skin fibroblasts exposed to adalimumab and cyclosporine A

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Abstract

Introduction: Adalimumab and cyclosporine A are drugs used in moderate to severe forms of psoriasis. Despite the molecular orientation of the drugs, there is a loss of adequate cell sensitivity to the anti-cytokine therapy.
Aim: To determine the changes in the gene expression profile associated with drug resistance in the culture of normal human dermal fibroblasts (NHDF) exposed to adalimumab or cyclosporine A compared to the controls.
Material and methods: NHDF was exposed to adalimumab/cyclosporine A for 2, 8, 24 h compared to the control culture. Molecular analysis was performed using mRNA and miRNA microarray techniques. The obtained results were analysed using PL – Grid infrastructure ($p < 0.05$).
Results: Of the 22277 ID mRNA, 47 are associated with drug resistance, of which the change in expression of 17 mRNA ID is statistically significant ($p < 0.05$). The greatest change in transcriptional activity (FC ≥ 1.3) was observed for *GLO1, SLC10A3, TUFT1, STATH, ABCB1, AGTR1*. Expression of these genes can be regulated by miR-199a-5p, miR-1231, miR-34a, miR-3188, and miR-106a (except *AGTR1)*.
Conclusions: The analysis of changes in the expression of mRNA and miRNA related to drug resistance gives the possibility of monitoring the effectiveness of anti-cytokine therapy.

Key words: psoriasis, drug resistance, personalized therapy, miRNA.

Introduction

Psoriasis is an inflammatory, chronic and recurrent autoimmune skin disease. The onset of the disease and the severity of skin lesions mainly depend on genetic factors, and behavioural and environmental factors also play an important role [1]. The histological reflection of clinical changes is epidermal hyperplasia, parakeratosis, hypogranulosis, neutrophil and lymphocyte infiltration in the epidermis and dermis together with dilated blood vessels [2]. At the molecular level, increased secretion of proinflammatory cytokines and adipokines is observed, including interleukin (IL) IL-1, IL-2, IL-6, IL-17, IL-12 and IL-23, interferon γ (IFN-γ), tumour necrosis factor α (TNF-α), and transforming growth factor β (TGF-β) [3–5]. Human dermal fibroblasts are an important model in in-vitro analyses that provide important new information on immunopathogenesis and the aetiology of drug resistance in personalized therapy. Fibroblasts are responsible for the production of connective tissue, moreover, they play an important role in the cell renewal system, maintaining skin integrity [6, 7]. One of the greatest achievements of modern medicine, giving a good chance to improve the quality of life of patients with moderate to severe psoriasis, in whom conventional treatment methods used so far (phototherapy, photochemotherapy, classic anti-psoriasis drugs – methotrexate, cyclosporine A (anti-IL-2 drug), acitretin, new low-molecular substances – apremilast, dimethyl fumarate) did not allow satisfactory effects to be obtained by the introduction of

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biological drugs with anti-cytokine activity [8, 9]. Among them, the recommended group of drugs is TNF-α inhibitors, which have become an important part of healthcare worldwide in the treatment of inflammatory diseases such as psoriasis [10]. This group includes adalimumab, which is a human monoclonal antibody that specifically binds to TNF-α, as a result of which the signalling pathways induced by said cytokine are blocked [11, 12]. Drug resistance is a well-known phenomenon that occurs when diseases become tolerant to pharmaceutical treatment [13]. Also in the case of anti-cytokine therapy, despite the molecular focus of treatment, the phenomenon of drug resistance is observed, so it is important to analyse the network of interrelationships between cytokines contributing to the intensification of inflammation, as well as to look for new, complementary molecular markers for detection of loss of adequate response to treatment [14] .

Aim

The aim of this study was to assess the effect of adalimumab on changes in the gene expression profile associated with drug resistance in Normal Human Dermal Fibroblasts (NHDF) cultures, *in vitro* stimulated with adalimumab at 8 µg/ml for 2, 8, 24 h or cyclosporine A at 100 ng/ml for 2, 8, 24 h compared to a non-drug-treated control culture. This will allow the selection of new transcripts and assessment of the possibility of their use as additional molecular markers in the diagnosis and detection of the lost response to an anti-cytokine therapy. In addition, it was assessed whether micro RNA (miRNAs) molecules could affect the expression of selected gene transcripts.

Material and methods

NHDF cell line (CC-2511; Lonza, Basel, Switzerland) was used to conduct the study. As recommended, the cells were cultured in a 5% CO_2 atmosphere at 37°C in a Direct Heat CO2 Incubator (Thermo Scientific, Waltham, MA, USA). FBM medium (Fibroblast Basal Medium; Lonza, Basel, Switzerland) enriched with hFGF-B (Human Fibroblast Growth Factor-basic), insulin and gentamicin (FGMTM SingleQuotsTM; Lonza, Basel, Switzerland). The experiment used NHDF cells between passage 4 and passage 6, with a viability of ≥ 98%. Cell culture was exposed to 100 ng/ml cyclosporine A and 8 µg/ml of adalimumab for 2 (H_2), 8 (H_8), and 24 (H_24) h compared to the untreated cells (control). Selected concentrations of both drugs correspond to their average therapeutic concentration in the blood serum of patients with psoriasis.

For isolation of total RNA, TRIzol® reagent (Invitrogen Life Technologies, California, USA) was used and extractions were carried out according to the protocol recommended by the manufacturer. The extracted genetic material was subjected to quantitative (GeneQuant II spectrophotometer; Pharmacia LKB Biochrom Ltd., UK) and qualitative (agarose electrophoresis; SUBMINI K. Kucharczyk T.E., Poland) analysis.

The evaluation of the expression profile of drug-related genes in NHDF cultures treated with drugs was performed using HG-U133A_2.0 oligonucleotide microarrays (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. The following stages can be distinguished in the analysis: preparation and labelling of ribonucleic acid samples, hybridization of samples with probes present on the microarray plate and interpretation of the obtained data. The obtained results were analysed using the PL-Grid software together with the GeneSpring 12.6.1 program ($p < 0.05$). Next, in order to select miRNAs that differentiate the anti-TNF drug-exposed culture from the control culture, a miRNA microarray expression profile (GeneChip® miRNA 2.0 Arrays; Affymetrix, Santa Clara, CA) was assessed. MirTAR tool (http://mirtar.mbc.nctu.edu.tw/human/predictionIndex.php) was used to indicate which of the selected miRNAs could potentially affect the expression pattern of the analysed genes. A detailed description of the individual stages of the microarray analysis has been previously described by us [7].

Results

Based on the Affymetrix NetAffxTM Analysis Center (http://www.affymetrix.com/analysis/index.affx) after entering the query of "drug resistance" (on 10/03/2019), out of 2227 mRNA IDs present on HG- U133A_2.0 microarray, 47 mRNA IDs were related to this phenomenon. In the first stage of evaluation of the microarray profile of the analysed genes, a one-way ANOVA variance analysis test with Benjamini-Hochberg correction was performed, which gave the opportunity to compare all analysed groups of transcriptomes compared to the control culture. In addition, it became possible to indicate which of the IDs mRNAs change their expression under the influence of adalimumab or cyclosporine A in a statistically significant manner ($p < 0.05$). Out of 47 mRNA IDs, in NHDF culture with adalimumab/cyclosporine A for 17 mRNA IDs observed statistically significant changes.

In the next stage, Tukey's post-hoc multiple comparison test was performed to obtain more detailed information on differences in mRNA expression in the analysed transcriptome groups. Based on the results obtained, the number of mRNA IDs differentiating the culture exposed to adalimumab compared to the control was the following H_2 vs. C = 6 mRNA IDs; H_8 vs. C = 9 mRNA IDs; H_24 vs. C = 1 mRNA ID. In turn, the number of mRNA IDs differentiating the culture exposed to cyclosporine A was the following H_2 vs. C = 7 mRNA IDs; H_8 vs. C = 4 mRNA IDs; H_24 vs. C = 4 mRNA IDs.

In NHDF culture exposed to adalimumab it can be observed that 1 IDs mRNA corresponding to the *DAP3* gene (↓) specifically differentiates NHDF culture 2 h after drug introduction compared to the control. In turn, in NHDF culture stimulated with cyclosporine A, it was observed that 5 mRNA IDs specifically differentiated the culture after 2 h: *BCRA3* (↑), *MTMR11* (↓), *TUFT1* (↓), *RRS1* (↑), *SRRT* (↓). In turn, increasing the exposure time to adalimumab to 8 h caused an increase in the number of mRNA IDs specifically differentiating the culture to 4 mRNA IDs that correspond to the genes: *RRS1* (↑), *SRRT* (↑), *MTMR11* (↓), *UVRAG* (↓). In addition, increasing the exposure time of fibroblasts to cyclosporine A to 8-hours showed 3 mRNA IDs specifically differentiating the culture: *UVRAG* (↓), *SLC30A5*(↑), *RIC8A* (↓).

In contrast, *SSRT* (↑) is also a transcript that specifically differentiates skin fibroblasts incubated for 24 h with an anti-TNF drug. In turn, *AGTR1* (↑) was characteristic for 24-hour exposure of cells to cyclosporine A. However, no mRNA IDs differentiating culture regardless of how long the cells were exposed to the drugs. It should be noted that the transcripts *GLO1* (↓↓), *SLC10A3* (↑↑), *TUFT1* (↑↓), *STATH* (↓↓), *ABCB1* (↑↑) are the genes that differentiate the culture together with adalimumab exposed for 2 and 8 h while *ATG14* (↑↑) and *AGTR1* (↑↑) are the genes that differentiate the culture together with cyclosporine A exposed for 2 and 24 h, and *TROVE2* (↓↓) for 8 and 24 h.

Table 1 shows changes in the direction of expression of genes differentiating culture depending on the time of exposure to adalimumab/cyclosporine A compared to non-incubated cell culture (1.3 < FC ≥ 1.3).

The last part of our work was associated with finding those micro RNA molecules (miRNAs) which might potentially influence the transcriptional activity of analysed genes (Table 2). The analysis of miRNA microarray indicated 20 miRNAs differentiating the cell culture after 2 h from adding the drug, compared with the control, but only for 4 miRNAs a potential impact on the expression of differentiating genes was observed. 8 hours' exposure showed that 12 miRNAs differentiated the fibroblasts culture, but for 3 miRNAs interaction with genes associated with drug resistance was confirmed. In turn, extending the incubation of cells with the drug to 24 h indicated 3 miRNAs differentiating the culture, but none of them had a potential effect on the expression profile of the analysed genes (Table 2).

Table 1. Changes in the transcriptional activity profile of the genes associated with drug resistance differentiating the NHDF culture exposed to adalimumab compared to the control culture (1.3 < FC ≥ 1.3)

	ID	Gene symbol	H_2 (up/down)	FC (H_2 vs. C)	H_8 (up/down)	FC (H_8 vs. C)	H_24 (up/down)	FC (H_24 vs. C)
NHFD+ adalimumab	205807_s_at	TUFT1	Up	+2.723	Down	–1.117	Up	–1.018
	209994_s_at	ABCB1	Up	+2.081	Up	+1.376	Down	–1.091
	204928_s_at	SLC10A3	Up	+1.582	Up	+1.637	Up	+1.162
	200681_at	GLO1	Down	–1.278	Down	–1.215	Up	–1.001
	206835_at	STATH	Down	–1.338	Down	–1.342	Down	–1.037
NHDF+ cyclosporine A	208016_s_at	AGTR1	Down	–1.186	Up	+1.059	Up	+1.526

(+) overexpression of gene (increased level of mRNAs); (-) suppressed gene expression (decreased level of mRNAs); ID – ID of the probe on a microarray; FC – fold change; C – control culture; H_2, H_8, H_24 time of exposure to the drug.

Table 2. The miRNA microarray analysis of NHDF cells treated with the drug compared with a control culture

Compared groups	ID	Name	FC	Change	Gene
H_2 vs. C	hsa-miR-199a-5p_st	hsa-miR-199a-5p	3.55	Up	*SLC10A3* *GLO1*
H_2 vs. C	hsa-miR-1231_st	hsa-miR-1231	2.24	Up	*GLO1*
H_2 vs. C	hsa-miR-34a_st	hsa-miR-34a	6.52	Up	*TUFT1*
H_2 vs. C	hsa-miR-3188_st	hsa-miR-3188	2.97	Up	*TUFT1*
H_8 vs. C	hsa-miR-3188_st	hsa-miR-3188	2.93	Up	*TUFT1*
H_8 vs. C	hsa-miR-1231_st	hsa-miR-1231	2.77	Up	*GLO1*
H_8 vs. C	hsa-miR-106a_st	hsa-mir-106a	2.45	Up	*GLO1*

Discussion

The premise for conducting these analyses was the observation of the loss of an adequate response to treatment during molecularly directed anti-cytokine therapy [15, 16]. Based on the microarray technique used, it was found that a relatively small number of gene transcripts are directly related to the phenomenon of drug resistance. For example, 341 mRNA IDs present on microarray plates used in our work are bound to the TNF-induced signalling pathway [17]. This may, therefore, suggest that the process of still losing sensitivity to treatment at the molecular level is an insufficiently understood topic. This is also indicated by our observation that, although aimed at assigning the genes selected in this work to specific signalling pathways based on the PANTHER program (protein annotation through evolutionary relationship) [18], it did not indicate the association of 5 mRNA IDs that most significantly changed the profile expression, to no signal cascade. On the other hand, however, the described situation creates the opportunity to perform further, in-depth analyses in the context of molecular mechanisms of response to treatment for several key genes.

Verstockt *et al.*, in their study on a group of 116 Crohn's disease patients treated with adalimumab, observed that some of them had drug resistance. They found this phenomenon at two critical points: 4 weeks after starting treatment and 12 weeks after the first dose. They indicate that the concentration of adalimumab in the initial period of therapy to some extent determines the subsequent potential benefits of TNF inhibitor treatment [19]. These conclusions confirm our observations carried out *in vitro*. The largest number of genes differentiating cultures with the drug from control was found after a short 2-hour exposure time, and then gradually decreased. In addition, also 2 h after adding adalimumab to NHDF cultures, the change in gene expression is the largest, and as time goes by and close to 24 h, the level of transcriptional activity of the selected genes was similar to that observed in the control culture of skin fibroblasts.

The largest increase in transcriptional activity was observed for the *TUFT1* gene coding for tuftelin, whose role has so far been discussed primarily in the context of enamel development and mineralization [20, 21]. Shilo *et al.* analysed the expression pattern of tuftelin at the mRNA and protein level at various stages of mouse embryo development. They observed the expression of tuftelin at the pre-tooth stage suggesting that it plays an important role in more than one cellular process. These researchers also found high dynamics of changes in the *TUFT1* level during the period of their observations [22], which to some extent is also confirmed by our observations. It is also worth noting that changes in the expression profile of *TUFT1* are associated with the activation of adaptive mechanisms by cells in response to hypoxia, and its potential role in the process of cell differentiation is emphasized [23]. However, considering the previously described lack of cytotoxic effect of adalimumab on NHDF culture [7], it can be assumed that the anti-TNF drug at a concentration of 8 µg/ml, which corresponds to the average therapeutic concentration of the drug in the serum of patients treated, does not induce hypoxia in the fibroblast microenvironment. Nevertheless, it is pointed out that hypoxia is the factor that induces *TUFT1* overexpression, which is an unfavourable prognostic factor. However, these observations have been made on a cancer model. They also indicate that the regulation of this gene is dependent on miR-671-5p, whose transcriptional activity is reduced in hypoxic conditions [24]. In turn, in our analysis of the miRNA microarray expression profile, we found that the expression pattern of *TUFT1* is potentially regulated by 3 miRNA molecules whose level was higher in adalimumab-exposed culture compared to control. At the same time, their overexpression after 2 h did not affect the expression of *TUFT1*, which was observable after extending the time of adalimumab exposure to skin fibroblasts to 8 h. The common miRNA molecule affecting *TUFT1* transcription activity for 2 and 8 h incubation is miR-3188, whose level remains substantially the same while silencing expression of the regulated gene transcript. Zhou *et al.* emphasize that an abnormal miRNA expression pattern may be associated with the induction of treatment resistance. Based on the conducted research, they indicated that miR-3188 is involved in the development of loss of cell sensitivity to fulvestrant treatment used in oncological therapy [25]. These observations in the context of the role of miR-3188 have also recently been confirmed by Zhao *et al.* They observed a significant impact of miR-3188 in the development of drug resistance on chemotherapeutic treatment, linking its occurrence to the effect of this miRNA molecule on signal transduction along the mTOR/PI3K/AKT/c-JUN pathway [26]. It cannot be excluded that the concurrent increase in expression of miR-34a and miR-3188, potentially regulating TUFT1 expression after 2 h of incubation of cells with a drug that did not translate into a decrease in TUFT1 transcript expression, may be found in our work due to the fact that miRNA molecules should not be considered only in the context of negative expression regulators, but also its enhancement. This seems justified as in some therapeutic strategies it is aimed to reduce the level of a given miRNA through anti-miRNA molecules, binding in terms of complementarity with miRNA [27].

Research conducted by Bobek *et al.* indicate that the role of tuftelin in particular biological and cellular processes depends on the type of cells [28]. This is confirmed by the observations of Shipeng *et al.* in an *in-vitro* fibroblast model where they observed an increase in *TUFT1* expression (FC = 2.4) in response to their exposure to low intensity red light [29]. Also in our analysis we found overexpression of *TUFT1* 2 h after the introduction

of the anti-TNF drug into culture, which, in conjunction with previous reports [7, 24, 28, 29] suggests that in fibroblasts under the influence of an external factor, the level of *TUFT1* will increase. Interesting, in the context of our work are the observations of Natsumi *et al.*, who emphasize the possibility of using changes in expression of *TUFT1* not only as a complementary molecular marker, but also as a new, attractive therapeutic target. They indicate that the expression pattern of the discussed gene and the encoded protein correlated with the response to anticancer treatment both *in vitro* and *in vivo* [30]. This observation indicates the usefulness of cell culture analyses and the possibility of referring *in vitro* results to *in vivo* analyses.

Also, the A*BCB1* gene can be doubled in expression after introduction of adalimumab into NHDF culture. The mechanism of action and the emergence of drug resistance with the participation of proteins encoded by this gene is the removal of xenobiotics with significant specificity for the substrate from the cell (the protein is an ATP dependent pump), as a result of which a decrease in drug concentration in the body is observed [31]. Observations of Boyer *et al.* indicate that higher *ABCB1* expression contributes to the induction of drug resistance. At the same time, they emphasize that the gene in question should not be directly linked to loss of response to treatment as silencing its expression did not significantly improve the results of pharmacotherapy [32]. Thus, *ABCB1* overexpression may indicate a group of patients in whom drug resistance should be expected. In this way, alternative therapy paths could be developed at the beginning of the implementation of a given therapeutic strategy, which emphasizes the need to develop and implement personalization of treatment, in which molecular analyses are extremely important [33]. The last of the genes for which an increase in transcriptional activity was observed was *SLC10A3*, which plays an important role in the transport of the drug into the cell [34]. Kirschmeyer *et al.* observed, like us, that the expression profile of the gene in question can be regulated by miRNAs. They found that miR-146b-5p reduced the number of mRNA *SLC10A3* copies [35], which was not confirmed by our research. Under the influence of the addition of adalimumab to culture, we have observed that hsa-miR-199a-5p potentially affects the expression of *SLC10A3*. However, it must also be noted that this miRNA molecule interacts with two gene transcripts: *SLC10A3* and *GLO1.* Perhaps, therefore, despite the potentially possible interaction of *SLC10A3*: miR-199a-5p, the complementarity of the resulting complex is small. Foulkes et al. suggest that the expression pattern of *SLC10A3* is dependent on TNF-α concentration observing a decrease in its activity with an increase in cytokine concentration [36]. Therefore, reducing TNF-α concentration by its neutralization with adalimumab resulted in the overexpression of *SLC10A3*, which was confirmed by the results obtained in this paper.

Analysis of the microarray profile of mRNA and miRNA for the selected genes showed that the *GLO1* gene can be regulated by three miRNA molecules: miR-1231, miR-106a, miR-199-5p. Regardless of time, we found a reduction in its expression in response to the addition of adalimumab to NHDF cultures. Glyoxalase 1 (GLO1; S-d-lactoylglutathione lyase, EC 4.4.1.5) is a part of the glyoxalase system, which, in addition to GLO1, consists of GLO2 (hydroxyacyl glutathione hydrolase, EC 3.1.2.6). This enzyme is responsible for the transformation of cytotoxic oxo-aldehyde, i.e. methylglyoxal (MGO) is d-lactate [37]. Hutschenreuther *et al.* indicate that overexpression of *GLO1* is characteristic of cancer cells in which excessive amounts of MGO accumulate. They also link the increase in expression of this gene with the induction of hypoxia in the tumour microenvironment [38]. Therefore, our silencing of *GLO1* expression under the influence of adalimumab suggests that this drug does not cause adverse changes in cell metabolism and its microenvironment, which in consequence could cause cells to acquire cancer. However, in the previous paper, we observed an increase in expression of the survivin-encoding gene, a protein that basically does not exist under physiological conditions [17]. This confirms the occurrence of complex changes and interrelationships between genes, and also constitutes a premise for further analyses, all the more that the analysis of changes in the *GLO1* expression pattern is associated with predicting response to treatment [39, 40]. We have observed that miR-1231 may affect *GLO1* expression after 2 and 8 h of exposure of dermal fibroblasts to adalimumab. The increase in miR-1231 expression is another premise for the safety of adalimumab and the relatively low risk of induction of carcinogenesis [41, 42].

In contrast, in the fibroblasts culture exposed to cyclosporine A, our results showed that the only gene associated with the phenomenon of resistance that meets the assumptions $1.3 < FC \geq 1.3$ is the gene *AGTR1* encoding Angiotensin II receptor sub-type 1. It is interesting that the highest differences in the expression profile of this gene was observed after 24 hours' incubation of NHDF culture with the drug, while under adalimumab treatment, the effect was observed earlier. Yamani *et al*. described that overexpression of *AGTR1* was associated with response to rejection after transplantation [43]. Observations by Ma *et al.* showed that *AGTR1* can be a useful supplementary marker and explained that overexpression of this gene was strongly associated with promoting epithelial-mesenchymal transition [44], what also showed that during cyclosporine therapy a higher risk of carcinogenesis might be observed [45]. These and our results suggest that during the cyclosporine A treatment, a drug resistance phenomenon may be observed, however its frequency is lower compared with the adalimumab therapy.

Nevertheless, it should be noted that these assumptions apply to skin fibroblasts, which were the research

model in our work. Therefore, in further analysis, we intend to determine changes in the expression of selected genes and miRNAs in the clinical material.

Conclusions

Adalimumab and cyclosporine A affect the transcriptional activity of genes associated with drug resistance. However, our analysis shows that the phenomenon of drug resistance is more pronounced with adalimumab than cyclosporine A. A thorough understanding and observation of the molecular mechanisms associated with the cellular response to adalimumab will help us better understand the complex nature of autoimmune diseases.

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Conflict of interest

The authors declare no conflict of interest.

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