Original paper

Effect of adalimumab on the expression of genes encoding TNF-α signal paths in skin fibroblasts *in vitro*

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Abstract

Introduction: Tumour necrosis factor (TNF-α) is one of the main cytokines participating in inflammation and immune response. Biological effects of the cytokine action, mediated by two receptors: TNFRSF1A and TNFRSF1B involve activation of many signal paths, thus change the transcription activity of many genes. The mechanism of action of an anti-TNF medicine consists in blocking TNF-α though preventing activation of signal paths.
Aim: To single out mRNA and microRNA genes relating to TNF-α signal paths, the expression of which could indicate sensitivity of cells to the medicine in question.
Material and methods: The material used in the research consisted in the cell line of regular human skin fibroblasts NHDF (CC-2511 Lonza, Basel, Switzerland) exposed to adalimumab with a concentration of 8.00 µg/ml of the medium for 2, 8 and 24 h, compared with the control material, i.e. non-stimulated cells. Molecular analysis was performed using the oligonucleotide expressive micro-matrices technology HG-U133A, miRNA 2.0 Array micro-matrices and RTqPCR.
Results: mRNA: BIRC5, MAP3K4, ZFAND5, JUN differentiate cells exposed to the anti-TNF medicine, regardless of the time of cell/medicine incubation. TNF-α transcription activity is reduced during exposure of NHDF cells to adalimumab. miRNA regulating transcription activity of the said 4 mRNA and miRNA related to TNF-α and its receptors was also singled out.
Conclusions: It was ascertained that adalimumab has therapeutic potential and affects genes engaged in signal paths activated by TNF-α. The results indicate the TNF-α usefulness as the molecular, supplementary marker in diagnostics and control of treatment effects.

Key words: TNF-α inhibitors, genes, *in vitro*, adalimumab, fibroblasts.

Introduction

Implementation of biological immunomodulatory medicines in the therapy of psoriatic arthritis (PsA) was an extraordinary achievement since it considerably improved the quality of life of the patients. However, the fact that these preparations have been introduced for therapy purposes relatively recently, still not enough information is available on their long-term action and potential possibility of generating tolerance and no response to long-term treatment.

It is known that tumour necrosis factor α (TNF-α) participates in the pathogenesis of autoimmune diseases. Recent research resulted in the formulation of inhibitors of the proinflammatory cytokine, which were used in the psoriasis and psoriatic arthritis therapy. Through TNF-α uptake, they block proinflammatory effects of its action, which is clinically manifested in the remission of symptoms [1].

TNF-α is popularly called tumour necrosis factor α [1, 2]. It belongs to the TNF protein superfamily and is a pleiotropic proinflammatory cytokine participating in the inflammatory and immunologic regulation of the human body [1–3]. Active TNF-α has a homotrimeric structure. It appears in the following forms: soluble

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TNF-α – sTNF and transmembrane TNF – tmTNF [3]. It is generated by many types of cells, in particular by macrophages, monocytes, T and B lymphocytes and synoviocytes. The gene for TNF-α is located on chromosome 6 in segment 6p23-6q12 among tissue compliance system genes. There are two types of receptors for TNF-α. These are proteins containing cysteine, membrane I-type – TNF-R1 (CD120a, p55/60) and TNF-R2 (CD120b, p75/80) [2]. Both molecular mass and the very nature of the transmitted signal are different from the above receptors. TNF-R1 is present almost in all nuclear cells of the human body and stimulated by free and transmembrane forms of TNF-α. TNF-R2 shows affinity for TNF transmembrane forms and can be found within fibroblasts, endothelial cells, lymphocytes and macrophages. Apart from membrane receptors, TNF-α binds with two soluble receptors: sTNFR55 and sTNFR75 [1–5]. While binding with specific receptors, tumour necrosis factor α activates them, which entails the whole series of events inside the cell (Figure 1). Induction of many signal paths may cause either activation or suppression of various biological processes, which confirms the multidirectional action of TNF-α. Through the connection with TNF-R1, the CASPASES route is activated and, finally, the apoptosis occurs and through the activation of the MAP KINASE route it participates in apoptosis, proliferation and inflammatory reaction. In turn, TNF-R2 receptor activates a cascade of reactions of the NF-κB route, inducing an inflammatory reaction in the cell [6].

When binding with TNF-α, adalimumab inactivates signalling cascades activated by receptors for TNF-α, thus hindering the inflammation. Our own clinical observations suggest transcription activity of genes encoding TNF and its receptors: TNFR1 and TNFR2 may constitute a marker of no stable effects of treatment, either during the application of traditional methods of general treatment or biological therapy.

Aim

Taking into account that molecular changes precede phenotypic changes, the aim of the paper was to assess the change in transcription activity of genes encoding TNF-α and its receptors: TNFRSF1A and TNFRSF1B and singling out transcriptomes relating to signal paths activated by TNF-α. It was also determined whether the observed changes may involve regulation of expression of these genes through selected miRNA (micro RNA) particles. The intention of the authors was to draw the attention to the possibility of using gene encoding proteins of signal paths activated by TNF-α as a supplementary diagnostic marker in pro-inflammatory diseases and determine the time after which it is possible to observe mo-

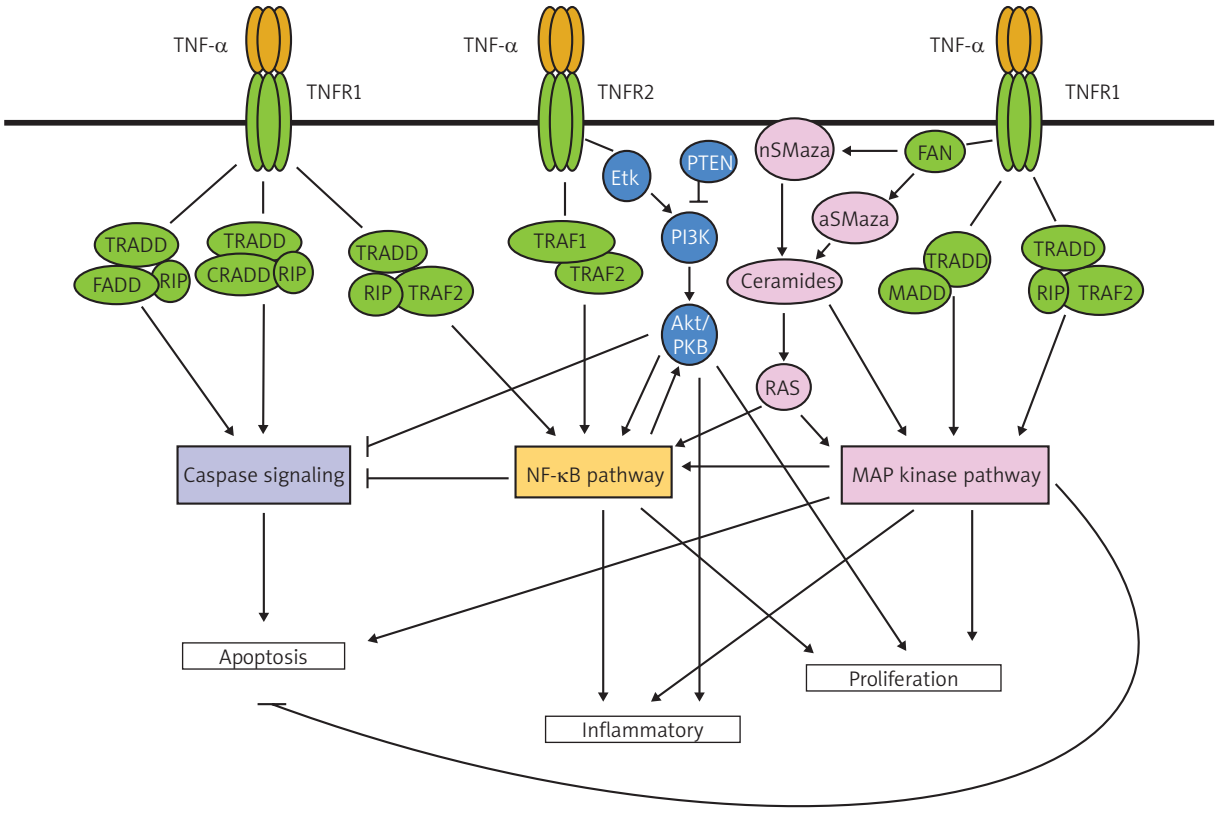

Figure 1. Signal paths activated by TNF-α

lecular changes in the *in vitro* system, after exposure of the cells constituting the research model to the biological medicine (adalimumab).

Material and methods

Cell culture

The material used in the research consisted in the cell line of regular human skin fibroblasts NHDF (CC-2511 Lonza, Basel, Switzerland). The cells were cultured in the atmosphere of 5% CO_2, at 37°C in Direct Heat CO_2 Incubator (Thermo Scientific, Waltham, MA, USA), in the FBM (Fibroblast Basal Medium; Lonza, Basel, Switzerland), enriched in hFGF-B (Human Fibroblast Growth Factor-basic), insulin and gentamicin (FGM™ SingleQuots™; Lonza, Basel, Switzerland).

NHDF cell stimulation

Changes in the expression profile of genes involving signal paths activated by TNF-α were tested in regular human skin fibroblasts NHDF, between passage 4 and 6. Having reached the sub-confluence (70%), adalimumab was added to the culture (concentration of 8.00 µg/ml) to the medium and was bred for 2 h, 8 h and 24 h. The culture was controlled against regular human skin fibroblasts NHDF, which were not exposed to adalimumab. NHDF cells with the life-span ≥ 98% were used for experiments. The cytotoxicity of the anti-TNF medicine was evaluated with the use of XTT test (In Vitro Toxicology Assay Kit, XTT based; Sigma-Aldrich, St Louis, MO, USA).

Extraction of total RNA

The molecular analysis was commenced with the extraction of total RNA from NHDF cells, with the use of TRIZOL® reagent (Invitrogen Life Technologies, California, USA), according to the producer's protocol. RNA was cleared with the use of RNease–Free Dnase Set Mini Kit (Qiagen GmbH, Germany).

The generated total RNA constituted the matrix to mark transcriptomes, with the use of expressive micro-matrices technology HG-U133A, according to the producer's instructions (Affymetrix).

Expressive micro-matrices

The mRNA transcriptome was determined in control and tested cells with the use of micro-matrices technology, basing on HG-U133A (Affymetrix Inc. California, USA).

Having labelled the mRNA with biotin, the received aRNA (amplified RNA) was hybridized with the probes on HG-U133A micro-matrices. The hybridization mixture was prepared with the use of GeneChip Hybridization, Wash, and Stain Kit (Affymetrix, Santa Clara, CA). Intensity of aRNA fluorescence was analysed with the use of Affymetrix GeneArray Scanner 3000 7G and GeneChip® Command Console® Software (Affymetrix, Santa Clara, CA).

The results of the matrix experiment were standardised with the use of *RMA* (Robust Multichip Average) program, based on logarithmic transformation of the mRNA fluorescence signal value ($\log_2$). Then, on the basis of the literature data and Affymetrix NetAffx™ Analysis Center database (http://www.affymetrix.com/analysis/index.affx), 341 mRNA genes were selected connected with the signal paths activated by TNF, which might be analysed with the use of micro-matrices technology using HG-U133A.

mRNA of genes differentiating skin fibroblasts (NHDF) exposed to adalimumab from the control sample were singled out on the basis of the analysis performed in Agilent GeneSpring program. The criterion to state the mRNA as differentiating required the $\log_2$ value of the difference of fluorescence signals between compared samples – fold change (FC) to be higher than 1.1, assuming the statistical gravity factor $p < 0.05$. The comparative analysis of singled out transcriptomes was performed with the use of platform for micro-matrices data integromic analysis (https://lifescience.plgrid.pl/).

miRNA microarray analysis

The analysis of the expression profile of miRNA which are responsible for regulation of histamine-related genes was performed using commercially available GeneChip® miRNA 2.0 Array (Affymetrix, Santa Clara, CA).

The first step comprised labelling of miRNA with biotin by polyadenylation and ligation. The evaluation of labelling efficiency was verified using ELOS QC Assay (FlashTagBiotin HSR RNA Labeling Kit, Affymetrix). Subsequently the hybridization of labelled RNA molecules of the microarray and probes were scanned using GeneArray Scanner 3000 7G (Agilent Technologies, CA). The scanned data were processed for signal values using Microarray Suite 5.0 software (Affymetrix).

Agilent GeneSpring GX software was used for statistical analysis of the data after microarrays scanning. Differentially expressed genes were determined using one-way ANOVA (analysis of variance) test with asymptotic computation of *p*-values. The criteria used for differentially expressed genes required the absolute value of fold change (FC) to be greater than 1 ($|FC| > 2$) in at least one compared sample pairs. The standard cut-off of *p*-value < 0.05 was set to determine statistical significance of mRNA fluorescent signals. Microarray data analysis was performed with the use of the GeneSpring 12.6.1 platform (Agilent Technologies, Inc., Santa Clara, CA, USA) and PL-Grid Infrastructure.

Quantitative real-time reverse transcription polymerase chain reaction assay (RTqPCR)

The matrices experiment was validated with the use of RTqPCR method. Changes were determined of the expression profile of TNF-α and its receptors: TNFRSF1A

and TNFRSF1B in regular human skin fibroblasts NHDF stimulated for: 2 h, 8 h and 24 h with adalimumab when compared with the control sample, i.e. non-stimulated cells. The reference gene was β-actin.

The reaction was conducted with the use of pairs of starters specific for the sequence of each tested gene. Oligonucleotide sequences of starters used for gene amplification: TNF-α (forward: 5' CTCAAGCTGAGGG GCAGCTCC 3', reverse: 5' TGGGTGAGGAGCACATGGGTG 3'), TNFRSF1A (forward: 5' CACCACAGTGCTG TTGCCCCT 3', reverse: 5'TGGAGTGGGACTGAAGCTT GGG 3'), TNFRSF1B (forward: 5'AGTATGG CCCCAGGGGCAGTACA3', reverse: 5' TCTCTCTGCAGGCACAAGGGCTT3'), ACTB (forward: 5'-TCACCCACACTGTGCC CATCTACGA-3', reverse: 5'-CAGCGGAACCGCTCATTGCCAATGG-3'). The RT-qPCR reaction was conducted with the use of Opticon™ DNA Engine Sequence Detector (MJ Research Inc., Watertown, MA, USA) and SYBR Green Quantitect RT-PCR Kit (Qiagen, Valencia, CA, USA), according to the producer's recommendations. The thermal profile of the reaction was as follows: reverse transcription: 50°C for 30 min, polymerase activation: 95°C for 15 min, 40 cycles composed of the following stages: denaturation: 94°C for 15 s, starters' activation: 53.3°C for 30 s, prolongation: 72°C for 45 s.

Statistical analysis

The statistical analysis of the results was performed with the use of the licensed version of Statistica 12 PL (StatSoft, Tulsa, Oklahoma, USA), assuming the statistical gravity factor $p < 0.05$.

The statistical analysis included performance of the parametric test – one-way analysis of variance ANOVA ($p < 0.05$) and then the post-hoc Tukey's test in order to determine the statistically significant differences in the expressions of tested genes between the individual times of exposure of NHDF cells to adalimumab.

Results

The experimental part of the paper was commenced with the XTT cytotoxicity test (In Vitro Toxicology Assay Kit, XTT based; Sigma-Aldrich, St Louis, MO, USA). The results showed no statistically significant variances between regular skin fibroblasts exposed to adalimumab and the control sample. In the experiment, adalimumab was added to the culture of 8.00 µg/ml of the medium (assuming that it is the average concentration of the medicine in the human body – a therapeutic dose).

The degree of mRNA transcriptome variance in regular human skin fibroblasts (NHDF) – control ones and those exposed to adalimumab for 2 h, 8 h and 24 h was determined as a result of the analysis of the concentration profile of 341 mRNA involving the TNF-α signal paths with the use of the hierarchical clustering method, using the Euclidean distances. The results of the analysis indicate that transcription activity of tested genes changes after the medicine has been added to the culture. Observed differences are the greater the shorter is the time of cell exposure to the medicine. The greatest variances in the expression profile of tested genes were observed 2 h after the anti-TNF medicine was added to the culture and the smallest – after 24 h.

The results indicate a great activity of cell adaptive mechanisms in response to the addition of adalimumab, an anti-TNF medicine, to the culture of skin fibroblasts. The next stage of comparative analysis involved an answer to the question which of the analysed mRNA particles may be categorised as the clusters differentiating the cells, regardless of the time of exposure of the fibroblasts to adalimumab. The analysis commenced with one-way ANOVA test, combined with the post-hoc Tukey's test (Table 1 A, B).

The generated results indicate that among 341 mRNA relating to TNF-α signalling, 171 mRNA differentiate the compared clusters of fibroblasts, assuming the value of $p < 0.05$. When increasing the criterion of the differentiation power to $p < 0.001$, gradual reduction in the mRNA differentiating the transcriptome clusters was observed – down to 66 mRNA (Table 1 A). Supplementing the analysis with the post-hoc Tukey's test, it was shown that among 171 mRNA singled out in the ANOVA test, the 2 h transcriptome clusters vs. C are differentiated by 122 mRNA, after 8 h vs. C – 85 mRNA and after 24 h vs. C – 8 mRNA (Table 1 B).

This stage of the analysis was finished with the generation of the VENNA diagram (Figure 2), which showed that regardless of the time of exposure of skin fibroblasts to adalimumab, assuming that $p < 0.05$, four mRNAs changed their transcription activity. These are the following: JUN, BIRC5, MAP3K4, ZFAND5 (Table 2).

The analysis of the expression profile of the four tested transcripts shows that the more the time of cell exposure to the medicine is prolonged, the smaller the difference in the transcription activity of those genes between the cells exposed to adalimumab and the control sample. Similar tendency was observed in case of changes in the mRNA TNF-α and its receptor TNFRSF1A concentration profiles between the control sample and the culture tested for TNF-α after only 8 h of exposure to the medicine and for TNFRSF1A only for 2 h of NHDF cells incubation with the medicine.

The matrix experiment was validated with the use of RTqPCR method and involved the analysis of changes in transcription activity of the following: TNF-α and its receptors: TNFRSF1A and TNFRSF1B, depending on the time of NHDF cells exposure to the stimulating factor, i.e. adalimumab (2, 8 and 24 h) compared with the non-stimulated cells, constituting the control sample. The results were presented as the quantity of mRNA copies in 1 µg of total RNA. Table 3 presents the results of the post-hoc Tukey's test, indicating statistically significant differences in the expression of genes: TNF-α, TNFRSF1A, TNFRSF1B

Table 1. The quantity of mRNA genes relating to signalling paths activated by TNF-α differentiating transcriptomes of NHDF control cells and those exposed to adalimumab. **A** – Determined with the use of the one-way ANOVA test in GeneSpring 11.5 (Agilent Technologies), depending on the differentiating power. **B** – Determined with the use of the post-hoc Tukey's test, indicating which mRNA from the ones singled out with the use of the ANOVA test may be used to differentiate the defined transcriptome clusters

A

***P*-value**		< 0.05	< 0.02	< 0.01	< 0.005	< 0.001
mRNA	341	171	135	123	104	66

B

	mRNA			
	2 h	**8 h**	**24 h**	**C**
2 h	171	133	119	122
8 h	38	171	83	85
24 h	52	88	171	8
C	49	86	163	171

in the culture of NHDF cells exposed for 2, 8 and 24 h to the anti-TNF medicine.

The next stage involved the analysis of micro-RNA (miRNA) regulating expression of JUN, MAP3K4, ZFAND5, BIRC5 transcripts, differentiating the cells exposed to adalimumab when compared with the control cells, regardless of the time of their exposure to the medicine. The first stage of the analysis involved the performance of ANOVA test, then – on the basis of the microRNA data base (www.microrna.org) and miRnada algorithm, it was searched which differentiating miRNA may potentially regulate expression of differentiating mRNA, set out in Table 2 B. All miRNA (Figure 3), which may potentially participate in the regulation of genes differentiating the cells, regardless of the time of their exposure to the medicine, showed overexpression when compared with the control sample. Moreover, it was ascertained, on the basis of miRNA SVR score that all of them may potentially regulate expression of singled out mRNA, however, the greatest likelihood of such interaction ranged between MAP3K4 and hsa-mir-27a (increase after 2 h: FC = 3.55) and hsa-mir-27b (increase after 2 h FC = 2.87 and 8 h FC = 2.09) and between ZFAND5 and hsa-mir-30d (increase after 2 h FC = 2.19 and 8 h FC = 2.55). After 24 h, the expression increased only for hsa-mir-132 (FC = 2.73), which may regulate the expression of ZFAND5 gene.

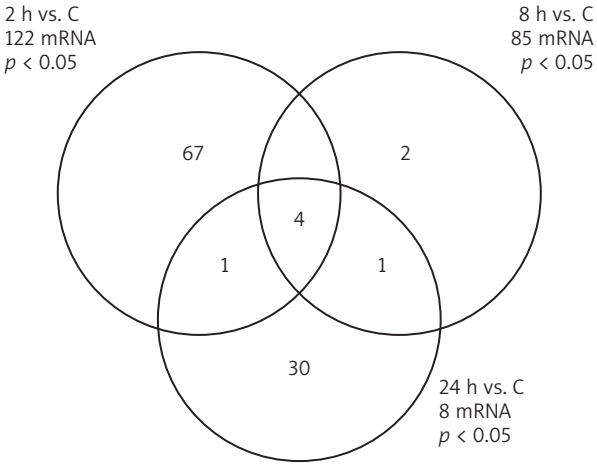

Figure 2. The VENNA diagram, depicting the quantity of specific mRNA differentiating clusters of skin fibroblasts transcriptomes (NHDF), depending on the time of exposure to the medicine

Discussion

Interaction of TNF-α with TNFRSF1A or TNFRSF1B receptors is directly linked to the activation of TNF-α signal paths [7–9].

One of the groups of biologically active medicines, focused on molecular mechanisms of a given disease, is anti-TNF medicines. The anti-TNF therapy involves the use of the following medicines: infliximab (chimeric anti-TNF-α antibody), etanercept (fusion protein) and adalimumab (human monoclonal anti-TNF-α antibody) [10].

In spite of the documented safety of using the biological medicines, one must take into account the reports describing potential dangers of the therapy involving biological medicines [11, 12]. Consequently, it is very important to monitor the efficacy of the therapies conducted with the use of molecular markers, which would make it possible to change the strategy of treatment before unfavourable effects occur, such as medicine resistance.

This paper makes an attempt to determine the adalimumab capacity to modulate the transcription activity of genes engaged in signal paths activated by TNF-α and assess the role of miRNA in controlling the transcription activity of the analysed genes. The research model in this

Table 2. A – mRNAs differentiating the cells exposed to adalimumab, regardless of the time of exposure to the medicine the transcriptome differentiation power – *p*-value; the size of observed difference FC (log2) – Fold change; a positive value of FC – overexpression of the gene when compared to the control sample, negative value – 11 suppression of gene expression when compared to the control sample. **B** – miRNAs regulating gene expression differentiating cells exposed to adalimumab, regardless of the time of exposure to the medicine ($p < 0.05$). The miRNAs for which the greatest likelihood of interaction with a given transcript was ascertained, are marked in bold

A

Probe Set	Gene symbol	*P*-value	$\log_2$ FC 2 h vs. C	$\log_2$ FC 8 h vs. C	$\log_2$ FC 24 h vs. C
201466_s_at	JUN	1.034×10^{-5}	1.51	1.19	1.12
204089_x_at	MAP3K4	8.388×10^{-9}	1.51	1.83	–1.11
210272_s_at	ZFAND5	3.835×10^{-10}	1.49	1.32	–1.04
202094_at	BIRC5	3.054×10^{-7}	–1.59	–1.38	1.15

B

Group	miRNA name	Fold change	miRNA SVR score	mRNA
2 h vs. C	**hsa-mir-27a**	3.55	–0.8785	MAP3K4
	hsa-mir-27b	2.87	–0.8785	MAP3K4
	hsa-mir-377	2.7	–0.1634	BIRC5
	hsa-mir-30d	2.19	–0.1096	JUN
			–0.8820	ZFAND5
	hsa-miR-16	2.17	–0.2442	MAP3K4
			–0.1634	ZFAND5
8 h vs. C	**hsa-mir-30d**	2.55	–0.1096	JUN
			–0.8820	ZFAND5
	hsa-mir-377	2.32	–0.1634	BIRC5
	hsa-mir-27b	2.09	–0.8785	MAP3K4
24 h vs. C	hsa-mir-132	2.73	–0.2283	ZFAND5

Table 3. Results of the post-hoc Tukey's test ($p < 0.05$) indicating the statistically significant differences in the expression of genes: TNF-α, TNFRSF1A, TNFRSF1B singled out with the use of the RTqPCR method of the NHDF cells exposed for 2, 8 and 24 h to adalimumab

mRNA	ANOVA $p < 0.05$	Time [h]	0	2	8	24
TNF-α	0.007	0		NS	0.016679	NS
		2	NS		0.028904	NS
		8	0.016679	0.028904		NS
		24	NS	NS	NS	
TNFRSF1A	0.00000	0		0.026917	0.000772	0.000483
		2	0.026917		0.000160	0.000160
		8	0.000772	0.000160		NS
		24	0.000483	0.000160	NS	
TNFRSF1B	0.00002	0		0.000532	NS	NS
		2	0.000532		0.000632	0.000194
		8	NS	0.000632		NS
		24	NS	0.000194	NS	

experiment was a line of regular human skin fibroblasts (NHDF), exposed for various time (2, 8, 24 h) to the anti-TNF biological medicine: adalimumab, which in *in vivo* conditions is administered subcutaneously.

The quantity of mRNAs differentiating the cells exposed to the anti-TNF medicine, when compared with the control sample, i.e. the non-stimulated cells, was reduced with the increased time of their exposure. However, regardless of the time of exposure, 4 mRNA: JUN, BIRC5, MAP3K4, ZFAND5 were always the genes differentiating the cells exposed to the medicine when compared with the control cells.

The BIRC5 gene encodes survivin, a protein belonging to the family of apoptosis inhibitors. The biological activity of survivin manifests mainly in phase G1/S of the cell cycle. Complex location of survivin (cytoplasm, cell nucleus) is reflected in its role during cell cycle regulation [13].

The results obtained, presented in the form of intensity of fluorescence signals relating to the stimulation of NHDF cells with adalimumab, indicate that that the biological medicine in question affects the cell cycle of the regular human fibroblasts. Initially, a decrease expression of BIRC5 gene was observed after 2 h since the medicine was administered *in vitro* to the body, however, longer exposure time causes a gradual increase in the gene transcription activity, with the level after 24 h slightly higher than in control cells.

Taking into account the fact that survivin basically does not appear in finally diversified cells of adults, and its increased quantity was observed during neoplasia [14, 15], a particular care should be exercised during the medicine administration.

Changes in transcription activity of BIRC5gene, coupled with an analysis of changes in the expression profile for TNF-α also confirm the anti-apoptotic properties of survivin. Activation of receptors for TNF-α involves the shift of survivin from the cytoplasm to cell nucleus, where survivin joins the anti-apoptotic CDK4 complex (cyclin-dependant kinase 4)/p21, which causes p21 protein dissociation. This way, the cell gets the can move to S phase of the cell cycle, and p 21 protein shifts to mitochondrion, where it joins pro-caspase 3, which blocks the receptor-dependant apoptosis for TNF-α [16].

Griffith *et al*. [17] analysed that the level of survivin affects the sensitivity of cells to apoptosis activated by the combination of TRAIL (tumour necrosis factor-related apoptosis inducing ligand) to TNF receptors, in such a way that over-expression of survivin increases cell resistance to TRAIL. Regulation of the sensitivity to the said factor through the anti-apoptotic protein involves its modulation of the activity of caspases 3 and 7. Observations made by the research team correlate with the observations made during the described experiment.

Results presented in relation to BIRC5 expression suggest that longer time of NHDF cell exposure to adalimumab involves recovery of proliferation capability on

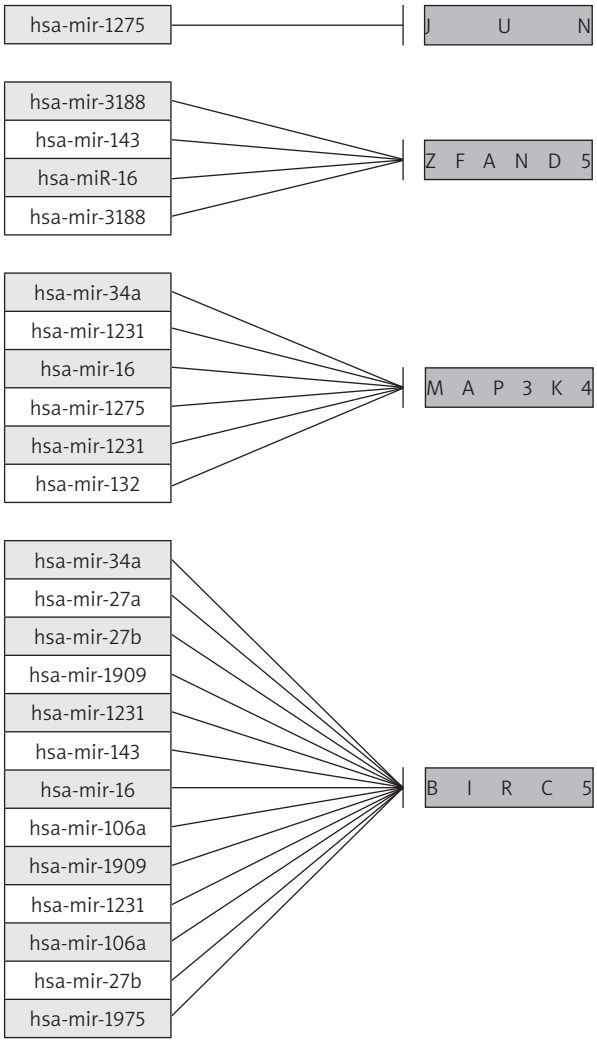

Figure 3. All miRNAs affecting the A transcription activity of 4 mRN genes differentiating the NHDF cells stimulated with the medicine from the control cells

the part of regular skin fibroblasts, thus suggest decreased reactive and blocking potential of TNF-α by the monoclonal antibody of adalimumab.

It was also observed that the medicine affects the transcription activity of the following genes: MAP3K4 and JUN, belonging to the MAP-KINASE family, which become activated during the three-stage phosphorylation.

Modulation of the transcription activity of MAP3K4 and Jun kinase shows the capability of adalimumab to affect proliferation and differentiation processes. These two genes encode proteins participating in the NF-κB signal path activated by TNF-α through their interaction mainly with TNFRSF1B receptor, which does not have death domains (DD) in its structure [1, 18, 19].

It was also observed that following the 2-hour exposure to the medicine, transcription activity of JUN and MAP3K genes is greater when compared with the control

culture. When compared with the control culture, after 24 h in case of JUN, the over-expression is still observed, though it is smaller than after 2 and 8 h. Whereas the 24-hour-long incubation of cells with the medicine suppresses the MAP3K4 gene, when compared with the control culture.

Fischer *et al.* [20] in the research conducted with the use of cell lines of gut epithelium: CaCo-2 and T-84 confirmed the participation of TNF-α in NF-κB-dependent signal paths and those involving activation of MAP kinases. They also observed that adalimumab affects the said signalling paths activated by TNF-α through suppressing the phosphorylation processed necessary to activate MAP kinases and generate an active form of iNF-κB.

Observations made on cell lines coincide with the ones made by Johansen *et al.* [21] on bioptats of psoriatic skin in patients treated with the anti-TNF medicine (adalimumab), as well as on the mouse model by Camara *et al.* [22].

The last of the common differentiating genes is ZFAND5, expressed in big quantities by brain cells, skeletal muscles. After 2 and 8 h, the over-expression of the gene is observed when compared with the control culture and after 24 h, ZFAND5 gene is suppressed. The role of this gene in muscle atrophy and osteoclast differentiation He *et al.* [23] confirmed the share of the said gene in the pathogenesis of inflammation and its role involving binding of ZFAND5 to ARE (Au Rich Elements) sequences, appearing at the end of the 5th gene encoding TNF-α and responsible for its final concentration. The bond between ZFAND5 and ARE sequence stabilizes mRNA of TNF-α [2].

That is why the analysis of the ZFAND5 role and expression profile of this gene in NHDF cells exposed to adalimumab indicates the efficacy of the anti-TNF therapy, since the expression of TNF-α is gradually suppressed along with the time of cell incubation with adalimumab.

Taking into account expression profiles of all 4 differentiating mRNAs, regardless of the exposure time, it may be assumed that during the anti-TNF therapy with the use of adalimumab on the model of regular human fibroblasts (NHDF), the cells aim to preserve the relatively stable environment conditions, a homeostasis. Reasons for this include the decreased transcription activity of proliferation-related genes under influence of a biological medicine, which is attempted to be balanced by increased gene expression encoding anti-apoptotic proteins. It suggests the necessity to regularly administer the medicine blocking the inflammation mediator in order to preserve clinically favourable and molecular effects. One must also remember the redundancy phenomenon, common for all cytokines, which translates into the possibility of affecting signal paths and inducing the biological effect despite blocking the TNF-α activity.

In this paper, it was also established which miRNA particles are responsible for the regulation of transcription activity of 4 mRNA, constituting the genes differentiating cells stimulated by the biological medicine from non-stimulated cells, regardless of the exposure time.

It was observed that all miRNA potentially participating in the expression regulation process of the 4 genes differentiating NHDF cells, regardless of the time of exposure to adalimumab, showed over-expression when compared with the cells non-stimulated by the biological medicine. It was determined that the highest likelihood of such impact occurs between MAP3K4 and hsa-mir-27a (increase after 2 h: FC = 3.55) and hsa-mir-27b (increase after 2 h FC 2.87 and 8 h FC = 2.09) and between ZFAND5 and hsa-mir-30d (increase after 2 h FC = 2.19 and 8 h FC = 2.55). After 24 h, the expression increased only in case of hsa-mir-132 (FC = 2.73), which may regulate expression of ZFAND5 gene.

It indicates the complexity of the gene expression regulation process, complexity of the factors leading to the occurrence of changes in the transcriptome expression profile. It also underlined the value of determination of changes in miRNA concentrations, since these particles considerably regulate gene expression. Then it becomes possible to analyse the changes in gene transcription activity more fully, providing for the complexity of transcriptome expression profile regulation.

The effect of miRNA in the process of gene transcription activity modulation during the anti-TNF therapy was confirmed in the observations made by Pivarcsi *et al.* [24]. It also showed that in the serum of patients treated with etanercept, constituting one of the TNF inhibitors, changes in the concentrations of 38 miRNAs particles were observed. In turn, Raaby *et al.* observed changes in miRNA concentrations regulating gene expression on bioptats of psoriatic skin in patients treated with adalimumab, as early as after 4 days of the anti-TNF therapy [25]. Moreover, the effect of post-transcriptionally regulating miRNA particles was confirmed in many pro-inflammatory diseases.

Krintel *et al.* observed than one of the miRNA particles regulating the TNF-α expression is hsa-miR-22, suggesting the possibility of using the miR-22 as the diagnostic marker in monitoring results of treatment with the use of adalimumab [26].

Origination of miRNA in RNA extract used in this research is not known. Isolation of total RNA involves the fact that all types of RNA particles are present in it. It is thus likely that in the extract, a certain cluster of miRNA particles originated from exosomes, i.e. cell-derived vesicles excreted probably by all types of cells, engaged in the process of intercellular communication. Exosomes contain numerous proteins, specific mRNA and biologically active miRNA [27]. Exosomes may be responsible for the loss of treatment efficacy. Moreover, they are used in the diagnostics and assessment of the staging of disease advancement [28].

Observations made over the years by numerous research teams indicate, on the one hand, the complexity

of processes TNF-α is engaged in and which it activates, however, on the other hand, they underline the usefulness of the use of the cytokine as the supplementary diagnostic marker for pro-inflammatory diseases.

Kacperska *et al.*, in the initial tests conducted on patients with multiple sclerosis (SM), which is an example of neurodegenerative disease, observed that TNF-α would be a useful serum marker reflecting the activity and stage of pathological advancement of processes accompanying SM. Nevertheless, they indicate the necessity to extend the research group and test the changes of TNF-α concentrations in the cerebrospinal fluid [29].

The possibility and usefulness of TNF-α in disease monitoring was also underlined by Olczyk-Kwiecień *et al.*, who observed a group of patients with rheumatoid arthritis (RA), emphasising the crucial role the cytokine plays in pro-inflammatory diseases, thus drawing attention to its potential use as a marker for diagnostic and monitoring of therapy [30]. Moreover, Komatsu *et al.*, on the basis of their own observations, stressed the possibility of monitoring the stage of the progress of inflammatory intestine diseases with the use of TNF-α as a marker [31].

In this paper, we observed the changes of concentrations of TNF-α and its receptors at the transcriptome level, as well as in genes relating to signal paths activating the said cytokine as early as after 2 h of exposure of the NHDF cells to an exogenic substance, i.e. – in this case – a biological anti-TNF medicine – adalimumab.

With reference to TNF-α, changes in its transcription activity are characterised by the fact that, regardless of the time of NHDF cells exposure to adalimumab, expression of the said gene is restricted. It constitutes another premise for a potential use of TNF-α as a supplementary marker to monitor pro-inflammatory diseases, as well as therapeutic effects. Changes in the TNF-α expression, in response to the biological medicine, as early as after 2 h of stimulation already shows that upon becoming a common medical and laboratory practice to determine the level of the said cytokine, the procedure would not be too much uncomfortable for the patients, since it would involve minimally longer, only 2 h' necessity to stay within the facility. Also the homogeneity of changes in the transcription activity would make it possible, basing on *in vitro* and then *in vivo* tests, to determine the range of correct values of the said cytokine, which would facilitate the interpretation of obtained results, at the same time preventing possible discrepancies.

Moreover, the results concerning changes in the TNF-α expression in NHDF cells exposed to the anti-TNF medicine, manifesting in reduced transcription activity with the time of medicine action, may confirm the efficacy and therapeutic potential of the biological medicine in question, used for the experiment.

Taking into account the significance of molecular changes and the awareness of their earlier occurrence than phenotypic changes, TNF-α would also constitute a useful indicator of the response to treatment, thus ensuring an opportunity to early detect the occurring resistance to medicine, which is unfortunately observed also in therapies conditioned by a given patient's genotype.

It will contribute to elaborate innovative diagnostic and therapeutic strategies, with particular attention to resistance to medicine and safety of therapy for the patient [32, 33].

Conclusions

Adalimumab, the anti-TNF medicine, shows therapeutic potential, manifesting itself in suppressing the TNF-α transcription activity and through modulating cytokine-dependent signal paths.

The significant role of TNF-α in the development and induction of inflammatory processes, as well as the development of personalised medicine, indicate that the pro-inflammatory cytokine could serve as the supplementary marker to be used in the diagnostics and therapy of pro-inflammatory diseases.

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Conflict of interest

The authors declare no conflict of interest.

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