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INFLUENCE OF CYCLOSPORIN A ON EXPRESSION PATTERN OF GENES ASSOCIATED WITH DNA REPAIR IN HUMAN DERMAL FIBROBLASTS

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Abstract: Cyclosporin A (CsA) is a cyclic nonribosomal peptide with immunosuppressive activity. Chronic immunosuppressive medication is associated with time distant side effects and is the cause of the different secondary diseases, including cancers (especially skin cancers). Anomalies in the functioning of DNA repair mechanisms are closely related to the processes of neoplastic transformation. The object of this study was to assess the impact of CsA exposure (8 h, early cell response) on expression of genes associated with DNA repair in normal human dermal fibroblasts (NHDF). NHDF from CC-2511 cell line were routinely maintained in FBM medium. Transcriptional activity of genes associated with DNA repair in NHDF after 8 h of cells exposition to CsA (C = 100 ng/mL) in relation to control cells was compared using Affymetrix HG–U133A 2.0 oligonucleotide microarray technique. GeneSpring GX fluorescence signals analysis of 1514 probes, which represented the expression of 875 genes selected from the NetAffx Analysis Center database for "DNA repair" query, demonstrated the inhibited expression of 32 probes (p-value < 0.05; Fold Change > 2.0), including: *BRCA1*, *RAD51*, *TOP2A*, *EXO1*, *RRM2*, *CDK1* and *POLE2*. The obtained results suggest that CsA can have a silencing effect on DNA repair genes. Therefore, the risk of skin cancer development during CsA therapy can result not only from immunosuppressive effects of the drug, but is also likely to arise from inhibition of DNA repair pathways.

Keywords: cyclosporin, DNA repair, NHDF, oligonucleotide microarray

Cyclosporin A (CsA) is a cyclic nonribosomal peptide composed of 11 amino acids, initially isolated from the fungus *Tolypocladium inflatum* (1). Immunosuppressive effects of CsA involve its binding to cyclophilin, a cytoplasmic protein of immunocompetent lymphocytes, especially T cells. CsA-cyclophilin complex is an inhibitor of calcineurin, a mediator of transcriptional activation pathway of interleukin 2 (IL-2). CsA-cyclophilin complex binds to calcineurin and prevents the dephosphorylation of the transcription factor NF-AT and its relocation to the cell nucleus, which excludes the increase in transcriptional activity of genes encoding IL-2 and related cytokines. CsA therapeutic activity takes place in the early phases of the cell cycle (G_0, G_1) and results from the inhibition of cellular and humoral immune responses and modification of inflammatory reactions. CsA affects the T_H cells activating process and thereby indirectly inhibits the production of antibodies and activation of macrophages. It also inhibits the B-lymphocytes to some extent (2–4). CsA also affects the mitochondria, preventing the opening of mitochondrial permeability transition pore – MPTP, thereby reducing the release of cytochrome C, which is an effective apoptosis-stimulating factor (5, 6).

CsA is widely used in patients after heart, kidney, liver, lung, pancreas, bone marrow, skin and small intestine transplants. Apart from transplant medicine, CsA is also used in psoriasis, severe atopic dermatitis, pyoderma gangrenosum, chronic autoimmune urticaria and, infrequently, in rheumatoid arthritis and related diseases, although it is only used in severe cases. In the United States, CsA is widely used in the form of eye drops for the treatment of dry eye (7). CsA was also studied for use as neuroprotective agent in case of traumatic brain injury. Experiments with animals demonstrated a reduction of damage associated with brain injury. It was shown that CsA blocks the formation of MPTP, which are responsible for the formation of neural tissue damage due to head injury or neurodegenerative diseases (8).

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Eukaryotic cells developed specialized detectors of genome damage. DNA repair requires efficient lesion detection, which is one of the major early cell responses to DNA damage. Induction of this process is accompanied by mechanisms that stop the cell cycle, which prolongs the time needed for DNA repair before replication and division of chromosomes. This ensures the restoration of genetic information and maintenance of gene sequence. Until now, six major DNA repair pathways in living cells were described, including direct reversal, base excision repair, nucleotide excision repair, mismatch repair, non-homologous end joining, microhomology-mediated end joining and homologous recombination, which are represented by expression of over 850 genes (9, 10). It is well recognized that anomalies in the functioning of DNA repair mechanisms are closely related to the processes of neoplastic transformation (11).

Little is known about CsA molecular mechanism of action, particularly about its influence on gene expression and responses induced at the cellular level. Side effects and multidrug resistance alteration are frequently observed during the treatment with CsA. Furthermore, chronic immunosuppressive medication is associated with time distant side effects and is the cause of different secondary diseases, including cancers (especially skin cancers). The object of this study was to assess the impact of CsA exposure (8 h, early cell response) on expression of genes associated with DNA repair in normal human dermal fibroblasts (NHDF).

EXPERIMENTAL

Cell culture

Human dermal fibroblasts from CC-2511 cell line (Clonetics, San Diego, CA, USA), routinely grown in fibroblast basal medium (FBM – Lonza, Basel, Switzerland), were used as the material for the study. Cultures were incubated under conditions of constant temperature of 37^{o}C, constant humidity of 95% and 5% CO_2 enriched atmosphere. NHDFs were exposed to CsA at a concentration of 100 ng/mL for 8 h. CsA concentration of 100 ng/mL complied with an average therapeutic concentration of the drug in blood plasma of routinely treated renal transplant patients (12). The study used a base solution of CsA in 0.9% NaCl (Novartis, Basel, Switzerland) at a concentration of 50 mg/mL.

Extraction of total RNA

TRIzol reagent (Invitrogen Life Technologies, California, USA) was used to isolate total RNA from samples, according to the manufacturer's protocol. Total RNA extracts were treated with DNase I (Fermentas International Inc., Ontario, Canada) and purified with the use of RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany), in accordance with manufacturer's instructions.

Qualitative and quantitative assessment of total RNA extracts

The quality of RNA was estimated by electrophoresis on a 1% agarose gel stained with ethidium bromide. RNA concentration was determined on the basis of absorbance at 260 nm using a GeneQuant pro RNA/DNA Calculator (Pharmacia LKB Biochrom Ltd., Cambridge, UK).

Oligonucleotide microarray gene expression analysis

To synthesize double-strand cDNA, 8 μg of total RNA was used (SuperScript Choice system; Invitrogen Life Technologies, CA, USA). The synthesis of biotinylated cRNA was carried out using BioArray HighYield RNA Transcript Labeling Kit (Enzo Life Sciences, New York, USA). Fragmentation of cRNA was performed with the use of Sample Cleanup Module Kit (Qiagen GmbH, Germany). Hybridization mixture was prepared using the GeneChip Expression 3'-Amplification Reagents Hybridization Control Kit and subjected to hybridization with HG-U133A 2.0 microarrays (Affymetrix Inc., California, USA). All steps were performed according to the Gene Expression Analysis Technical Manual (Affymetrix Inc., California, USA). Fluorescence intensity was measured with the use of Affymetrix GeneArray Scanner 3000 7G (Affymetrix Inc., California, USA).

Statistical analysis and prediction of differentially expressed genes

Six microarray plates were used: three for control and three for treated cells. For the analysis of NHDF transcriptomes, influenced by CsA exposure, among 22 277 mRNA probes present on the HG-U133A 2.0 microarray, transcripts associated with DNA repair were selected. The names of the probes were obtained from Affymetrix NetAffx Analysis Center database (13) after entering the query: "DNA repair". There were 1514 probes filtered, which represented the expression of 875 different genes.

Agilent GeneSpring GX software was used for statistical analysis of the data after microarrays scanning. Simultaneous normalization with RMA algorithm (Robust Multichip Average) of 22 277 fluorescence signal values for the six HG-U133A

2.0 chips was performed. Microarray quality control tests were carried out using: 3D Principal Component Analysis, analysis of the normalized fluorescence signal values for hybridization control probes and the 3'/5' ratios for internal controls. Differentially expressed genes were determined using T test unpaired and Fold Change (FC) filtering. P-values were computed using asymptotic method with 300 permutations and corrected using Benjamini-Hochberg multiple testing correction method. The standard cut-off of p-value < 0.05 was set to determine statistical significance of mRNA fluorescent signals. The criterion used for differentially expressed genes required the absolute value of FC to be greater than 2.0 (|FC| > 2.0) between compared samples.

RESULTS AND DISCUSSION

Cyclosporin A is known to inhibit nucleotide excision repair (NER) *via* downregulation of the xeroderma pigmentosum group A and G proteins, which is mediated by calcineurin inhibition (14, 15). The inhibition of NER mechanism is widely recognized to contribute to the skin cancer proneness in organ transplant patients. Also, CsA can induce DNA double-strand breaks (16) and it was shown that calcineurin inhibitors decrease DNA repair and apoptosis in human keratinocytes following ultraviolet B irradiation (17). Together, this suggests the potential role of CsA in carcinogenesis, which cannot be explained by immunosuppression alone.

Oligonucleotide microarray technique allows a detailed analysis of changes in transcriptional activity of tens of thousands of genes simultaneously. This technique is often used to study the impact of drugs on the cellular processes at the molecular level (18). Such study allows to perform the prediction of differentially expressed genes and also may help to understand how therapeutic agents modify the expression of specific genes of interest (19).

This study focused on investigating the transcriptional activity of genes associated with DNA repair in NHDF cells cultured in the presence of CsA for 8 h in comparison with control cultures. The use of HG-U133A 2.0 Affymetrix oligonucleotide microarray technology allowed to determine the gene expression parameters after obtaining normalized expression values for the individual probes. Comparison of gene expression in treated and control NHDFs was performed using Agilent GeneSpring GX software. Based on Affymetrix NetAffx Analysis Center database (13), the sets of probes associated with the pathway of interest were

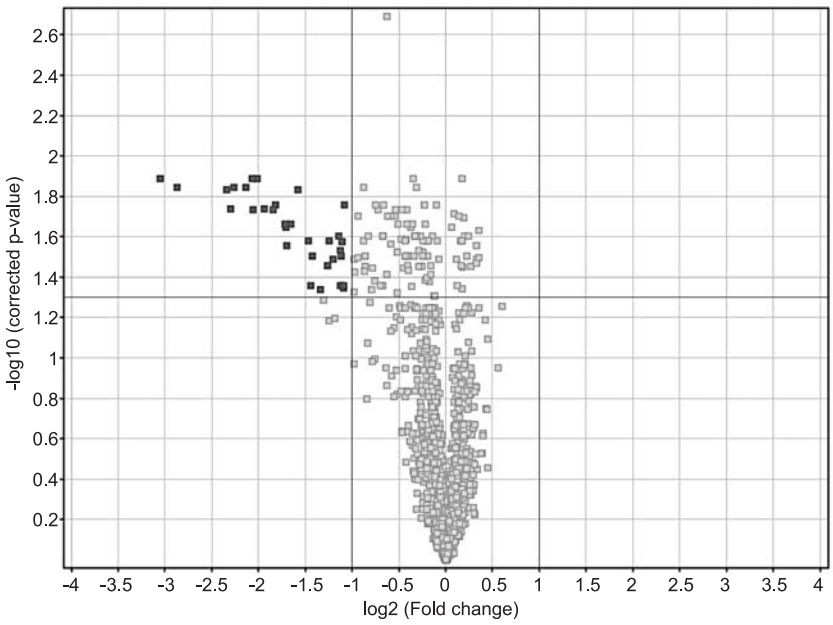

Figure 1. Volcano plot showing relationship between p-value (–log10 of p-value, y-axis) and fold change (x-axis, FC) as a result of T Test unpaired (1514 probes, n = 3). P-values were computed using asymptotic method with 300 permutations and corrected using Benjamini-Hochberg multiple testing correction method. Differential genes ($p < 0.05$; $|FC| > 2.0$) associated with DNA repair are shown in dark gray

Table 1. Probes for genes associated with DNA repair ($p < 0.05$; $|FC| > 2.0$), differentiating early cell response (8 h) of human dermal fibroblasts exposed to cyclosporin A (C = 100 ng/mL; n = 3) in relation to control cells.

Probe set ID	Gene symbol	Corrected p-value	FC Absolute	Regulation
201890_at	*RRM2*	0.0129	8.30	down
209773_s_at	*RRM2*	0.0143	7.33	down
210559_s_at	*CDK1*	0.0147	5.09	down
204126_s_at	*CDC45*	0.0181	4.94	down
203214_x_at	*CDK1*	0.0143	4.80	down
202503_s_at	*KIAA0101*	0.0143	4.39	down
203213_at	*CDK1*	0.0129	4.21	down
201291_s_at	*TOP2A*	0.0184	4.16	down
201292_at	*TOP2A*	0.0129	4.05	down
220651_s_at	*MCM10*	0.0181	3.85	down
204603_at	*EXO1*	0.0184	3.60	down
204146_at	*RAD51AP1*	0.0174	3.54	down
209642_at	*BUB1*	0.0216	3.30	down
206632_s_at	*APOBEC3B*	0.0225	3.28	down
218355_at	*KIF4A*	0.0278	3.26	down
204822_at	*TTK*	0.0216	3.16	down
220085_at	*HELLS*	0.0147	2.99	down
203145_at	*SPAG5*	0.0262	2.77	down
212949_at	*NCAPH*	0.0435	2.72	down
204033_at	*TRIP13*	0.0312	2.69	down
213007_at	*FANCI*	0.0458	2.54	down
208079_s_at	*AURKA*	0.0349	2.40	down
216237_s_at	*MCM5*	0.0262	2.37	down
219502_at	*NEIL3*	0.0323	2.30	down
205733_at	*BLM*	0.0249	2.22	down
203554_x_at	*PTTG1*	0.0292	2.19	down
204128_s_at	*RFC3*	0.0435	2.19	down
205909_at	*POLE2*	0.0312	2.18	down
204531_s_at	*BRCA1*	0.0266	2.16	down
213008_at	*FANCI*	0.0435	2.14	down
205024_s_at	*RAD51*	0.0448	2.14	down
202589_at	*TYMS*	0.0174	2.13	down

filtered. The aim of the analysis was to search for differences and similarities in the expression patterns of selected sets of genes. The set of 1514 probes, representing the expression of genes associated with DNA repair, in the form of values of the fluorescence signals, was used to determine differentially expressed genes characterized by the most significant fold change under the influence of CsA. T test unpaired, with a predetermined cut-off of p-value < 0.05, allowed to predict 128 mRNA probes,

Table 2. Genes associated with DNA repair, differentiating the response of NHDF exposed to CsA for 8 h.

Gene symbol	Encoded protein name
APOBEC3B	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B
AURKA	aurora kinase A
BLM	Bloom syndrome, RecQ helicase-like
BRCA1	breast cancer 1, early onset
BUB1	BUB1 mitotic checkpoint serine/threonine kinase
CDC45	cell division cycle 45
CDK1	cyclin-dependent kinase 1
EXO1	exonuclease 1
FANCI	Fanconi anemia, complementation group I
HELLS	helicase, lymphoid-specific
KIAA0101	KIAA0101
KIF4A	kinesin family member 4A
MCM10	minichromosome maintenance complex component 10
MCM5	minichromosome maintenance complex component 5
NCAPH	non-SMC condensin I complex, subunit H
NEIL3	nei endonuclease VIII-like 3 (E. coli)
POLE2	polymerase (DNA directed), ε 2, accessory subunit
PTTG1	pituitary tumor-transforming 1
RAD51	RAD51 recombinase
RAD51AP1	RAD51 associated protein 1
RFC3	replication factor C (activator 1) 3, 38 kDa
RRM2	ribonucleotide reductase M2
SPAG5	sperm associated antigen 5
TOP2A	topoisomerase (DNA) II α 170 kDa
TRIP13	thyroid hormone receptor interactor 13
TTK	TTK protein kinase
TYMS	thymidylate synthetase

associated with DNA repair, with significantly altered transcriptional activity level in NHDF cells exposed to CsA compared to the control group. Filtering the absolute values of fold change between compared cells was performed in order to narrow down a set of differentially expressed genes. Probes with |FC| > 2.0 (minimum two-fold increase or decrease in signal intensity) were selected and formed a panel of 32 transcripts. Differentially expressed probes are shown in Figure 1 in the form of Volcano plot, describing relationship between p-value and FC as a result of statistical analysis.

Analysis of transcriptional activity of genes involved in DNA repair mechanisms, differentiating early NHDF response to CsA exposure in relation to control cultures, showed that all typed transcripts were inhibited, including: *BRCA1* (2.16-fold), three probes for *CDK1* (5.09-, 4.80- and 4.21-fold, respectively), *EXO1* (3.60-fold), *POLE2* (2.18-fold), *RAD51* (2.14-fold), two probes for *RRM2* (8.30- and 7.33-fold, respectively) and two probes for *TOP2A* (4.16- and 4.05-fold, respectively). Detailed data, illustrating the influence of CsA, including probe set IDs, symbols of genes, p-values, FCs and regulation are shown in Table 1. Names of the encoded proteins for differentially expressed genes along with gene symbols are presented in Table 2.

Under the influence of CsA, the most inhibited gene was *RRM2* encoding ribonucleotide reductase small subunit M2. This reductase catalyzes the formation of deoxyribonucleotides from ribonucleotides. Synthesis of the encoded protein (M2) is

regulated in a cell-cycle dependent fashion. It was recently shown that *RRM2* may be a facilitating factor in colorectal tumorigenesis and UV-induced DNA damage repair (20).

RAD51 encodes recombinase from RAD51 family, which members are highly similar to bacterial RecA and *Saccharomyces cerevisiae* Rad51. These proteins are known to be involved in DNA repair and homologous recombination. *RAD51* protein product can interact with the ssDNA-binding protein RPA and RAD52, and it is thought to play roles in homologous pairing and strand transfer of DNA. It was also found to interact with BRCA1 and BRCA2, which may be important for the cellular response to DNA damage (21).

BRCA1, which was also notably influenced by CsA, encodes a nuclear phosphoprotein involved in maintaining genomic stability and also acts as a tumor suppressor. The encoded protein interacts with other tumor suppressors, DNA damage sensors and signal transducers to form a large multi-subunit protein complex known as the BRCA1-associated genome surveillance complex (BASC) (22). *BRCA1* protein product cooperates with RNA polymerase II and also influences histone deacetylase complexes through its C-terminal domain. Thus, this protein plays a role in transcription, DNA repair of double-stranded breaks and recombination (23).

CONCLUSIONS

Together, the results suggest that CsA can have a silencing effect on DNA repair genes. Therefore, the risk of skin cancer development during CsA therapy can result not only from immunosuppressive effects of the drug, but is also likely to arise from inhibition of DNA repair pathways.

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REFERENCES

1. Tedesco D., Haragsim L.: J. Transplant. 2012, 1 (2012).
2. Colombo D., Ammirati E.: J. Biol. Regul. Homeost. Agents 25, 493 (2011).
3. Voskamp P., Bodmann C.A., Koehl G.E., Tensen C.P., Bavinck J.N., Willemze R., Geissler E.K., de Gruijl F.R.: Transplantation 96, 871 (2013).
4. Wimmer C.D., Angele M.K., Schwarz B., Pratschke S., Rentsch M., Khandoga A., Guba M. et al.: Transpl. Int. 26, 999 (2013).
5. Elrod J.W., Wong R., Mishra S., Vagnozzi R.J., Sakthievel B., Goonasekera S.A., Karch J. et al.: J. Clin. Invest. 120, 3680 (2010).
6. Wilkinson S.T., Johnson D.B., Tardif H.L., Tome M.E., Briehl M.M.: Oncol. Lett. 1, 227 (2010).
7. Tu E.Y., Rheinstrom S.: Dry eye, in Ophthalmology. Yanoff M., Duker J.S. Eds., Mosby Elsevier, St. Louis 2008.
8. Sullivan P.G., Thompson M., Scheff S.W.: Exp. Neurol. 161, 631 (2000).
9. Menck C.F., Munford V.: Genet. Mol. Biol. 37, 220 (2014).
10. Maginn E.N., de Sousa C.H., Wasan H.S., Stronach E.A.: Biochim. Biophys. Acta 1846, 45 (2014).
11. Herman-Edelstein M., Rozen-Zvi B., Zingerman B., Lichtenberg S., Malachi T., Gafter U., Ori Y.: Biomed. Pharmacother. 66, 111 (2012).
12. Abd A.Y., Aziz N.A., Hassan Y., Salih M.R.M., Ghazali R.: Int. J. Pharm. Sci. 2, 224 (2010).
13. http://www.affymetrix.com/ (accessed on 02.05.2014).
14. Kuschal C., Thoms K.M., Boeckmann L., Laspe P., Apel A., Schön M.P., Emmert S.: Exp. Dermatol. 20, 795 (2011).
15. Han W., Soltani K., Ming M., He Y.Y.: Cancer Prev. Res. (Phila) 5, 1155 (2012).
16. O'Driscoll M., Jeggo P.A.: Bone Marrow Transplant. 41, 983 (2008).
17. Yarosh D.B., Pena A.V., Nay S.L., Canning M.T., Brown D.A.: J. Invest. Dermatol. 125, 1020 (2005).
18. Aleksander E., Hibner G., Zajdel A., Paul-Samojedny M., Wilczok A.: Farm. Przegl. Nauk. 10, 47 (2010).
19. Hibner G., Aleksander E., Zajdel A., Paul-Samojedny M., Wilczok A.: Farm. Przegl. Nauk. 1, 35 (2011).
20. Lu A.G., Feng H., Wang P.X., Han D.P., Chen X.H., Zheng M.H.: World J. Gastroenterol. 18, 4704 (2012).
21. Nogueira A., Assis J., Catarino R., Medeiros R.: Pharmacogenomics 14, 689 (2013).
22. Knudsen N.Ø., Andersen S.D., Lützen A., Nielsen F.C., Rasmussen L.J.: DNA Repair (Amst) 8, 682 (2009).
23. Li M., Yu X.: Cancer Cell 23, 693 (2013).