Short Communication

Analysis of Altered Gene Expression Profiles in Retinoic Acid or CpG-Treated Sprague-Dawley Rats with MNU-induced Mammary Adenocarcinoma by cDNA Macro Array

S. Mostböck[1,2], D. Macejová[1], M. Baranová[1], R. Weiss[2], S. Scheiblhofer[2], T. Verwanger[3], B. Krammer[3], J. Thalhamer[2] and J. Brtko[1]

[1] *Institute of Experimental Endocrinology, Slovak Academy of Science, Bratislava, Slovakia*
[2] *Institute of Chemistry and Biochemistry, University of Salzburg, Hellbrunnerstr. 34, A–5020 Salzburg, Austria*
[3] *Institute of Physics and Biophysics, University of Salzburg, Hellbrunnerstr. 34, A–5020 Salzburg, Austria*

Abstract. In the present work the effects of 13-cis retinoic acid (RA) and CpG-containing oligodeoxynucleotides (CpG-ODN) on the gene expression profile of spleen and tumor tissue in a MNU-induced mammary gland carcinoma rat model were investigated by the use of a commercial cDNA macro array (Atlas rat toxicology array 1.2, Clontech). Treatment with these components, either alone or in combination, induced differences of the expression profiles between the distinct treatment groups in both tissues. The large number of genes with altered expression (>200) points to a highly complex process *in vivo*.

Key words: Retinoic acid — CpG — MNU — cDNA array — Tumor

1-methyl-1-nitrosourea (MNU) is a well characterized carcinogen that induces adenocarcinomas in rat mammary gland with high specificity. This model has been proven to be of resemblance to human breast cancer and is therefore of great interest for tumor studies.

Using this experimental system, we recently have investigated the effects of CpG oligodeoxynucleotides (CpG-ODN) and 13-cis retinoic acid (13cRA) on tumor burden of MNU-treated Sprague-Dawley rats (Macejova et al. 2001). Retinoids have been demonstrated as chemopreventive agents in tumor treatment of chemically induced mammary gland carcinogenesis in both mice and rats (Moon and

Correspondence to: Julius Brtko, PhD., D.Sc., Institute of Experimental Endocrinology, Slovak Academy of Science, Vlárska 3, 833 06 Bratislava 37, Slovakia
E-mail: Julius.Brtko@savba.sk

Constantinou 1997) and are already used as treatment for advanced thyroid cancers in humans (Boerner et al. 2002). They are also involved in immunological processes *in vivo* as was indicated by our work dealing with the influence of different immune response types on the level of retinoic acid receptor expression (Brtko et al. 2000). Furthermore, as an alternative treatment we selected the application of CpG-ODN, because these immunomodulatory DNA motifs represent a strong "danger" signal for the innate immune system and act as a Th1-type adjuvant *via* the induction of type-1 interferons like IFN-α/β, IL-1β, TNF-α, IL-6 and IL-12 (Sun et al. 1998).

For the experiment, groups of female Sprague-Dawley rats were given 50 $mg \cdot kg^{-1}$ MNU on their 53^{rd}, 82^{nd} and 102^{nd} day of age. From the 55^{th} day of age, the first group of rats received 13cRA (1 $mg \cdot kg^{-1}$) intragastrically three times *per* week until the end of the experiment. The second group of rats received two intradermal injections of CpG-ODN (5′-GCTAGACGTTAGCGT-3′, 100 μg) and the third group a combination of these two treatments. A fourth group served as control, receiving MNU without any further treatment. The experiment was terminated when the rats reached their 146^{th} day of age. The results elicited a significantly beneficial effect of CpG-ODN treatment and a clear tendency to decrease tumor burden by RA treatment. Surprisingly, the combination of RA and CpG-ODN treatment abrogated the beneficial effects of the single approaches (Macejova et al. 2001).

In the present study we focused our interest on the gene expression patterns of tumors and spleens of these groups. For evaluation we utilized a commercial cDNA nylon array to screen for expression changes in a selected group of 1176 genes (Atlas Rat Toxicology 1.2 Array, ClonTech – selected for their relevance in tumors and immunological pathways) which were spotted onto a positively charged nylon membrane. The use of such cDNA macroarray allows handling in hybridization bottles and similar equipment as commonly used for Northern blot technique and no special equipment like a robot unit is required. Sample labelling is performed by radioactive nucleotides thus enabling the use of conventional film material for detection (Chalifour et al. 1994). The major drawback of this approach is the need for larger amounts of sample, the use of radioactivity for detection and the smaller number of spots, i.e. smaller number of genes that may be tested in one hybridization compared to high density DNA microarrays.

We selected tumor and spleen as the target tissue in order to address two major questions: (i) does immunological (CpG-ODN) and hormonal (RA) modulation (both revealed a tumor suppressive effect) induce different target genes in the tumor tissue and (ii) how does the immune system (in our case represented by the spleen) react to this two different types of treatment.

For this purpose, RNA was prepared from tumor and splenic tissue by the use of TriZol (GibcoBRL/Life Technologies) following the manufacturers' instructions. The RNA of each group was pooled and treated with DNAse I (Promega). The subsequent preparation was performed following the instructions given in the cDNA array kit (Atlas Rat Toxicology 1.2 Array, ClonTech), with the exception of the use of ^{33}P-CTP (Amersham Pharmacia Biotech) instead of labelled ATP and no

use of sheared salmon testes DNA. The pooled RNA was transcribed to radioactive labelled cDNA using the kits primer-mix, and hybridized to the cDNA arrays for 2 days at 65 ℃. After washing, the bound radioactivity was measured by exposure of the cDNA arrays to a BAS-MS imaging plate (Fuji) for three days and subsequent reading of this plate by a BAS 1800 II Reader (Fuji). The arrays were stripped and reused. To minimize possible deviation of the results by the methodology used, four cDNA arrays were performed in parallel. For each hybridization, a freshly prepared cDNA probe of each group was hybridized onto a cDNA array. During four consecutive hybridizations the probes were hybridized to each of the four arrays. First, the four tumor RNA pools were hybridized onto the cDNA arrays, then the four spleen RNA pools. Thus, each probe had been hybridized to each array, and each array was hybridized with eight probes. The amount of radioactivity, i.e. the amount of bound cDNA, was evaluated by analyzing the BAS reader image with an AIDA 2D quantification image analyzer software (Raytest, Germany) by placing square areas of the same size around each spot and additionally 56 areas in empty areas of each array as background control. Then, the absolute data obtained were normalized for each filter and hybridization in separate by substracting the average of the background areas from each spot and subsequent division of the values by the average of five housekeeping genes spotted on the cDNA arrays (polyubiquitin, GAPDH, cytoplasmic β-actin, tubulin α-1, ribosomal protein S29). The values from the four hybridizations of each sample were averaged and genes with an expression value above the average of background plus 3 times the standard deviation of the background spots were analyzed. Genes that were at least either two-fold over- or under-expressed, or were expressed above the background in one group compared to another group (which itself was not expressed with values higher than background), were counted as being differently expressed. Table 1 shows the number of genes with altered gene expression of each group in comparison with each other group in tumor and splenic tissue.

For further analysis, the expression data of the tumor samples were subjected to cluster analysis utilizing a Vector Xpression software (Informax, Frederick, Maryland, USA). Clustering was focused on the change of the expression, and several different clustering algorithms were employed. The results obtained point to interesting differences between the treatment groups, like a downregulation of genes belonging to general protein metabolism processes in treated groups *versus* untreated MNU control and an upregulation of nuclear proteins and vitamin metabolism in the RA treated group. Application of CpG obviously activated a number of genes involved in immune response mechanisms and apoptosis and the combinational treatment with RA and CpG led to an increased expression of genes participating in tumor development as well as induced cell death.

Summing up, using this standard procedures for the evaluation of different gene expression by array technology we found a set of genes in both tumor and splenic tissue whose expression levels are changed upon treatment with RA and/or CpG-ODN. The vast number of differentially expressed genes points to complex processes as a consequence of RA and/or CpG-ODN treatment, which is in contrast to an

Table 1. Number of genes with altered expression levels between different groups in the tissues tested

		Tumor			Spleen		
		2↑	2↓	+	2↑	2↓	+
control *vs*	RA	8	2	37	11	1	36
	CpG	18	1	27	10	4	39
	RA + CpG	19	1	21	6	13	17
RA *vs*	Control	2	8	70	1	11	81
	CpG	3	2	10	2	4	51
	RA + CpG	5	2	5	1	13	13
CpG *vs*	Control	1	18	145	4	10	72
	RA	2	3	95	4	2	39
	RA + CpG	1	0	31	3	15	15
RA + CpG vs	Control	1	19	150	13	6	139
	RA	2	5	101	13	1	90
	CpG	0	1	42	15	3	104

For each treatment group the change of gene expression was tested against each other group. Two-fold differences are therefore displayed twice (once as two-fold rise in a *vs* b, once as two-fold decrease in b *vs* a). 2↑, genes with an expression level at least two-times higher than in the compared group; 2↓, genes with an expression level at least two-fold lower; +, genes with an expression level above background *versus* genes expressed with background values in the compared group.

earlier work with the aromatase inhibitor Vorlozone. This study elicited differential expression of only 19 genes as evaluated by a cDNA microarray (Wang et al. 2001).

Further work will concentrate on validation and investigation of selected clusters to evaluate the possible interactions of gene families in tumor biology and altered genetic pathways induced by different ways of treatment.

In general, two different principles of tumor treatment are represented by the gene clusters: the influence on the tumor itself on one hand and a strong stimulus to the immune system on the other hand. Both aspects may help to find interesting links and possible targets for new hormon- and/or immunotherapeutic approaches for treating mammary gland adenocarcinomas.

Acknowledgements. This work has been supported by the Centre of Excellence grant No. ICA1-CT-2000–70008 and in part by the VEGA grant 2/2070/22 and the Austrian Krebshilfe Salzburg.

References

Boerner A. R., Petrich T., Weckesser E., Fricke H., Hofmann M., Otto D., Weckesser M., Langen K. J., Knapp W. H. (2002): Monitoring isotretinoin therapy in thyroid cancer using 18F-FDG PET. Eur. J. Nucl. Med. – Mol. Imaging **29**, 231—236

Brtko J., Hartl A., Weiss R., Bernhaupt A., Scheiblhofer S., Mostbock S., Thalhamer J. (2000): DNA immunization *in vivo* down-regulates nuclear all-trans retinoic acid receptors in mouse spleen cells. Mol. Cell. Endocrinol. **165**, 107—113

Chalifour L. E., Fahmy R., Holder E. L., Hutchinson E. W., Osterland C. K., Schipper H. M., Wang E. (1994): A method for analysis of gene expression patterns. Anal. Biochem. **216**, 299—304

Macejová D., Dvorčaková M., Weiss R., Scheiblhofer S., Mostböck S., Líška J., Szabová E., Zórad S., Thalhamer J., Brtko J. (2001): Reduction of 1-methyl-1-nitrosourea-induced mammary gland carcinoma by *in vivo* application of immunostimulatory CPG motifs in Sprague-Dawley rats. Gen. Physiol. Biophys. **20**, 445—448

Moon R. C., Constantinou A. I. (1997): Dietary retinoids and carotenoids in rodent models of mammary tumorigenesis. Breast Cancer Res. Treat. **46**, 181—189

Sun S., Zhang X., Tough D. F., Sprent J. (1998): Type i interferon-mediated stimulation of t cells by cpg DNA. J. Exp. Med. **188**, 2335—2342

Wang Y., Hu L., Yao R., Wang M., Crist K. A., Grubbs C. J., Johanning G. L., Lubet R. A., You M. (2001): Altered gene expression profile in chemically induced rat mammary adenocarcinomas and its modulation by an aromatase inhibitor. Oncogene **20**, 7710—7721

Final version accepted: February 19, 2003