cDNA-microarray analysis as a research tool for expression profiling in human peripheral blood following exercise

Running head : Exercise and microarray analysis

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Abstract

Exhausting endurance exercise has strong effects on the immune system. Changes have been shown in the cellular composition of peripheral blood and in gene expression within those cells. In this study, custom-made cDNA microarrays focused on inflammation were used to analyze gene expression blood cells obtained from eight half-marathon runners before (t0), immediately after (t1) and 24 hours after exercise (t2).

The microarrays that were used contained 384 different cDNAs spotted in triplicate. Differentially-regulated gene expression was analyzed using a simple rule-based clustering. Comparing t1 vs. t0, and t2 vs. t0, 36 and 21 sequences respectively, showed a consistent pattern of changes in all eight athletes. Taken together, the pattern of these modified genes can be viewed as a "gene expression fingerprint" for each time point in response to a half marathon. The known and novel genes identified here represent targets for further molecular characterization of the complex reaction of the body to an exhaustive challenge. These data suggest that gene expression fingerprints can serve as a powerful research tool to design novel strategies for diagnosis and treatment of exercise related injury and stress.

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Introduction

cDNA microarrays are widely used tools for comprehensive analysis of gene expression. Microarrays make it possible to evaluate large numbers of genes and to assess the pattern of gene regulation simultaneously in one tissue sample (6). In addition to identifying new target genes, this method also allows the clustering of genes according to similar patterns of expression and/or function. Cluster analysis aggregates all relevant genes according to their similar expression profile. With this information, it is possible to determine a collection of genes to define an expression fingerprint. In this context, the term "fingerprint" refers to a group of genes that display a similar pattern of expression that is associated with a specific physiological condition or genotype. In such a pattern, individual gene responses relative to other genes are the central element necessary for the recognition of the pattern. Even in complex multivariate systems, the pattern can be detected against a complex background of changes, and thus the operation of complex physiological responses can be recognized, even in the presence of other processes. Importantly, this means that specific patterns of expression can be used for diagnosis of diseases or to identify altered physiological states.

Gene expression fingerprints have become a useful tool in research and diagnosis, with multiple applications. For example, this technique can be used in the classification of different tumor types (4), identification and characterization of gliomas (18), disease classification in malignant pleural mesothelioma (14), molecular characterization during breast cancer development, diagnosis and treatment (21), defining the molecular pathogenesis of segmental glomerulosclerosis (20) and detecting exposure to toxic substances (12).

Similarly, we propose that gene expression fingerprints may provide information for the characterization and understanding of physiological and metabolic regulatory pathways of the responses to endurance exercise. The advantage of this method is its sensitivity. Although some relevant genes are not strongly regulated during exercise and therefore may be easily overlooked, the sensitivity of gene expression fingerprints enhances the chances of detecting such genes. Training and competition lead to immune changes in athletes. The immune response to exercise is a coordinated reaction of multiple organ systems (2). In blood, several different systems are activated or regulated following exercise, and involve diverse genetic responses (10; 15; 16; 24; 28). At present, there is little knowledge about the exact molecular mechanisms by which exercise affects the immune system.

In humans, peripheral blood is the most practical tissue with which to perform measurements of stress and immunological parameters at the cellular and molecular level. Using spotted cDNA microarrays, we examined blood samples of trained athletes before and after a half marathon (21.1 km) to look for changes in gene expression patterns. The genes represented on the cDNA microarray were selected based on knowledge about their role in inflammation, apoptosis, stress responses and related pathways.

In our first microarray data analysis, we used very stringent statistical parameters, and focused on highly significant differential expression events. It was necessary to use stringent statistics in order to minimize false positive find-

ings; however, this approach may also increase the risk of false negatives. Accordingly, we discovered some interesting candidate genes (30). In an effort to explain or support the observations made in this study, where possible we provided background data on the genes in question.

The aim of the present follow up study was to obtain a molecular fingerprint of gene expression in response to sustained exercise. We wanted to find a pattern of genes that was regulated similarly in all eight athletes, even if they were not significantly altered. Therefore, less strict statistical requirements were adopted. We used a system of clustering based on function to organize the expressed genes into biologically relevant groups (signal transduction, membrane proteins, cell interaction, apoptosis, anti-/ oxidative system, immune system and anti-/ inflammation).

Material and Methods

Material and Methods were described in detail by Zieker et al. (30). All values are presented as means ± standard deviation.
In short :

Eight well-trained male athletes (38.9 ± 11.8 years, BMI 23.6 ± 1.8 kg/m^2) performed an official half-marathon under competition conditions (21.1 km). None of the athletes suffered from acute or chronic diseases, or reported intake of medication, including antioxidants and nicotine abuse. The run started at 10:00 A.M. on a cool and humid December day (1°C), and took place on a hilly and demanding course. Blood samples (5 x 2.5 ml whole blood) were drawn in PAXgene™ Blood RNA Tubes (Qiagen, Hilden, Germany) at rest before (t0), immediately (up to 15 min) after (t1), and 24 h (t2) after exercise, in a sitting position. The individuals had been engaged in specific endurance training for at least 2 years (52.2 km ± 25.5 km/week, running).

We compared the gene expression pattern of whole blood cells in each athlete before and after exhaustive exercise.

Each subject gave written informed consent prior to participation in the study. The experimental protocols were approved by the Institute's Human Ethics Committee according to the principles set forth in the Declaration of Helsinki of the World Medical Association.

Preparation of glass cDNA microarrays
In our study, we used a cDNA microarray containing 1152 cDNAs. Insert cDNAs of all clones were amplified and the quality of the PCR products was routinely checked on an agarose gel stained with ethidium bromide prior to spotting. Each PCR sample was spotted together with positive and negative controls in triplicate. Isolation, amplification and labeling of RNA

Blood was drawn in PAXgene™ Blood RNA Tubes (Qiagen). RNA was isolated using the PAXgene™ Blood RNA Kit (Qiagen, Hilden, Germany) including a DNase I treatment according to the manufacturer's recommendations. The quality of the RNA was checked with a Lab-on-a-Chip total RNA nano biosizing assay (Agilent Technologies, Palo Alto, United States). Amplification was performed exactly according to the Eberwine procedure (27) using Ambion's MessageAmp™ Kit (Ambion Inc., Austin, USA). After in-vitro-transcription, the

indirect labeling method with amino-allyl was used, with equal amounts of all samples. The protocol follows exactly the TIGR protocol (Hasseman et al., Aminoallyl labeling of RNA for microarrays, http://pga.tigr.org/sop/M004.pdf).

Hybridisation of glass cDNA microarrays
Hybridization was done at 55°C for 14 – 16 h in a water-bath. To compare the gene expression pattern of each athlete before and after exhausting exercise we hybridized each slide with samples from t0 versus t1, and from t0 versus t2. A dye-swap was done with each investigation to compensate for any potential artifacts associated with the labeling process.

Scanning, feature extraction and analysis,
Signal and Feature extraction and Normalization
Scanning, feature extraction and analysis, Signal and Feature extraction and Normalization were performed as described elsewhere (30).

Genetic Fingerprint
Genes were clustered based upon similarity in gene expression from each slide with samples from t1 and t2 versus t0, respectively. The assumption for a gene to be clustered was that its regulation was identical in all eight athletes at the particular time.

Software
All parts of the feature extraction and analysis procedures were computed using the software program R Versions 1.8.1 and 2.1.0 (17) with a mixture of Bioconductor structures (7) and self written functions.

Results

The average time required to complete the half marathon was 105 ± 21 min. Using stringent parameters, we had identified eight transcripts with statistically significant responses to exercise ($p<0.05$). Differential regulation of six of these and some further genes of interest which had shown a strong trend had been confirmed by Realtime PCR. These results were recently described by Zieker et al. (30).

In this paper we focus on the identification of a gene expression pattern which is characteristic for this type of exercise. This approach allowed us to identify "expression fingerprints" for the particular time points after run.

Using a microarray containing 1152 cDNAs, we detected 36 genes which changed in the same direction in all eight athletes immediately after exercise (t1) compared to pre-exercise (t0) (Figure 1 and Table 1). All modified genes and their allocation to functional groups are summarized in Table 1. The PCR-evaluated genes and the genes which were altered at both sampling points are indicated. The up- or down-regulation of the respective genes is presented in Figures 1 and 2. When t2 is compared with t0 we detected 21 genes that were altered in a similar manner in all eight athletes at the particular time in the same direction (Figure 2 and Table 1). These genes are associated with signal transduction, membrane proteins, cell interaction, apoptosis, anti-/ oxidative system, immune system and anti-/ inflammation processes (see Table 1).

Table 1: Allocation of changed genes to functional groups

Gene-Clustering of t1 versus t0		Gene-Clustering of t2 versus t0	
Signal transduction / Membrane proteins / Cell interaction / Apoptosis		***Signal transduction / Membrane proteins / Cell interaction / Apoptosis***	
Gene-ID	**Gene Session-ID**	**Gene-ID**	**Gene Session-ID**
APP	AI827546	ADRB2	R08445
Beta 2 rec.	R08446	Caspase 2	AA481283
Caspase 5	W60703	Chemokine lig. 4 #	N57175
CD3E	H25061	Chemokine rec. 1	BC028078
CD3E antigen	H25061	Chemokine rec. 4	AA479357
CD81 * °	H30366	EDG1 #	W21494
Chemokine lig. 4 #	N57175	GRIA1	BF739768
EDG1 #	W21494	IGF2	H78540
ICAM2 *	R21644	Integrin beta 4	R48163
IL-18 R1	AA482489	SMPD1	H85744
Integrin alpha 4	BX091768		
Integrin alpha x * °	N64384		
MAPKAP K2 * °	R71819		
Phospholipase C	AA041245		
S12	H72224		
Selectin L * °	H00756		
Anti-/ Oxidative system		***Anti-/ Oxidative system***	
Gene-ID	**Gene Session-ID**	**Gene-ID**	**Gene Session-ID**
GSTM3 * °	R63065	GSS	R40853
GST pi	AA416781	SOD1	W25024
HSPB1	R33761	TRX R1	H15481
Peroxiredoxin 5	H20248		
Thioredoxin * °	R83156		
Thioredoxin 2	N32554		
Immune system		***Immune system***	
Gene-ID	**Gene Session-ID**	**Gene-ID**	**Gene Session-ID**
CD1C * °	BX116983	CD19 #	AI217645
CD8	W07753	IL-2 rec. beta #	R62939
CD14	R15360		
CD19 #	AI217645		
CD79B	R72128		
CD244 *	AI363168		
CSF 3 rec. (gran)	R31999		
IL-2 rec. beta #	R62939		
Integrin alpha M	AI307732		
Anti-/ Inflammation		***Anti-/ Inflammation***	
Gene-ID	**Gene Session-ID**	**Gene-ID**	**Gene Session-ID**
15-PGDH	AI832179	CD2	AI131504
IL-1 rec. antag. * °	AA497054	IFNGR1	H11838
IL-1 rec. type 2 #	H78484	IRAK1	R83860
Protein C #	BX107014	IL-1 rec. type 2 #	H78484
PTGS1	AA454668	MGST2	AA122237
		Protein C #	BX107014

* indicates genes, which were significantly regulated comparing t0 vs. t1 ($p<0,05$) by microarray analysis and were evaluated by Real Time PCR (30).
° indicates genes, which were confirmed by Real Time PCR with $p<0,05$.
# indicates genes, which are regulated in t0 vs. t1 and t0 vs. t2.

Discussion

Since the development of the microarray technique in 1995 (19), our knowledge of gene expression data from multiple organisms and tissues has increased greatly. This progress may largely be attributed to the fact that microarray technology permits researchers to monitor expression of large numbers of genes in a single experiment. To process and interpret these data, powerful computational approaches and bespoke algorithms have become necessary.

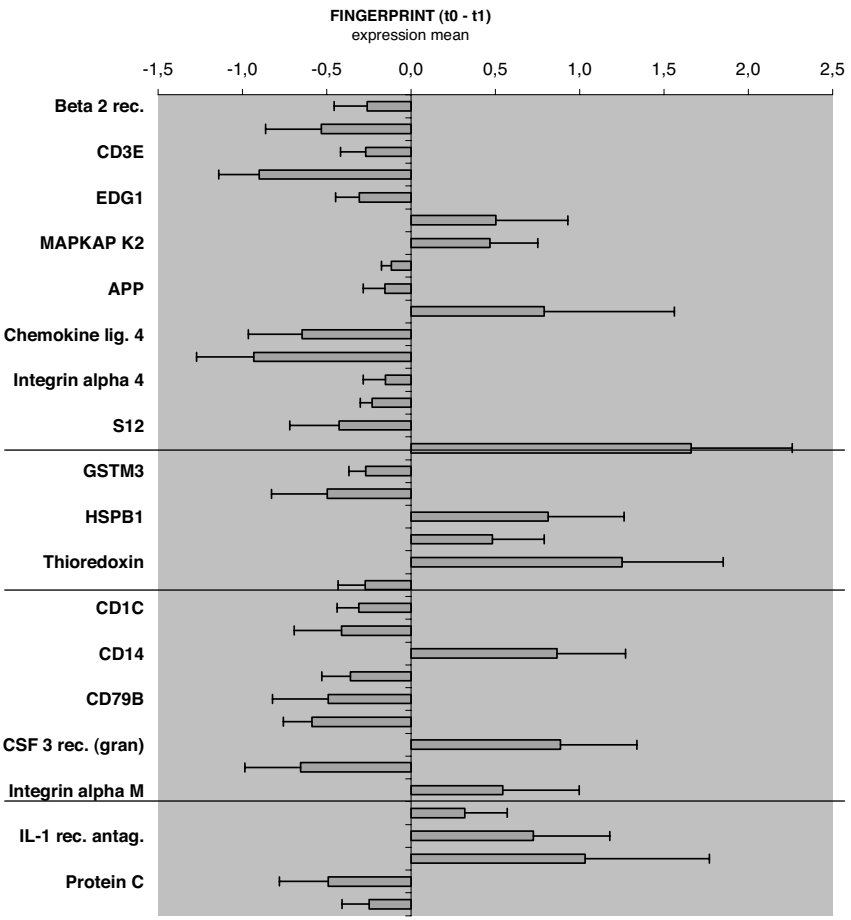

Figure 1: Genes were clustered based upon similarity in gene expression from each slide with samples from t1 versus t0.

By acquiring expression data for large numbers of genes, it is possible to find clusters of genes which have similar expression patterns that may be associated with coordinate regulations potentially due to involvement in the same or similar biochemical pathways (13). Such a pattern of similar regulated

genes at a certain condition or time point is called a "gene expression fingerprint" for this condition.

The most common techniques used for gene clustering in the microarray field are those reported by Eisen et al. (5), Törönen et al. (26) and Tamayo et al. (25), who performed dendrograms, self-organized maps and mathematical analysis for cluster analysis of gene expression data. In contrast, Hartuv et al. (8) have developed a new clustering algorithm, based on a graph theory approach clustering cDNAs by their oligonucleotide fingerprints. All these methods offer different possibilities to examine microarray data by clustering.

Specific gene analysis allows the detection of genes that can differentiate between various kinds of samples, e.g. genes that are differentially regulated in exercise and non-exercise samples or in different types of exercise. The expression level of these specific genes can be used to separate biological samples from each other.

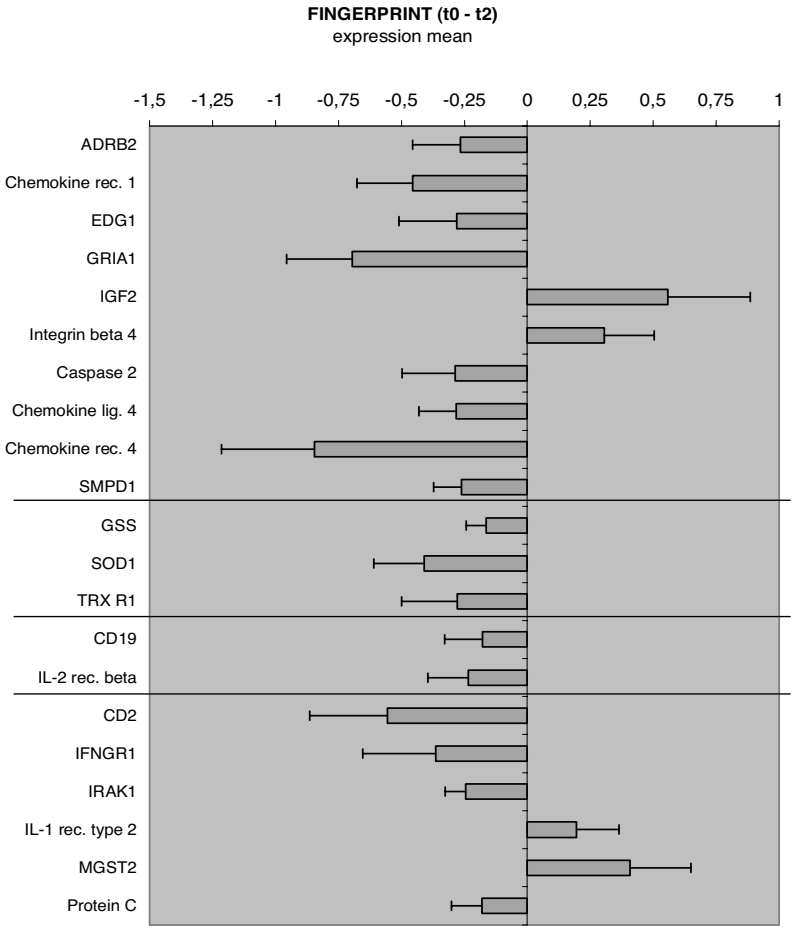

Figure 2: Genes were clustered based upon similarity in gene expression from each slide with samples from t2 versus t0.

Additionally, responses of relevant genes, that are not strongly regulated under the conditions of interest, can contribute to improve the clarity of the fingerprint.

The fingerprint of cancer cells has already become a strategy for identification, classification, characterization or treatment control in different tumor types such as leukaemia, central nervous system, melanoma, uterine, lymphoma, ovarian, breast and lung cancer (4), gliomas (18), pleural mesothelioma (14) and breast cancer (21). Fingerprints are also used to define the molecular pathogenesis of segmental glomerulosclerosis (20) and demonstrating exposure to different toxic substances (12),

Similarly, we performed a gene expression profiling in human peripheral blood following exercise using a stress-gene focused microarray. The aim of the recent investigation was to find a characteristic exercise-related fingerprint. Genes were clustered based upon similarity in alterations of gene expression between the respective sampling points. Our cluster analysis revealed 36 similar changed genes when t1 was compared with t0, and 21 genes when t2 was compared with t0. The genes were up- or down-regulated identically in all eight athletes. These patterns at the respective sampling points may be assigned as typical half-marathon-associated fingerprints. The respective genes were clustered into groups with specific functions, including signal transduction, membrane proteins, cell interaction, apoptosis, anti-/ oxidative system, immune system and anti-/ inflammation. Only a few of the designated fingerprint genes showed significant changes. Their potential function in exercise physiology has been discussed in detail previously (30). It is likely that exercise also modifies the expression of genes related to other functions in the body; there is certainly scope for further studies to broaden our knowledge of alterations in other genes after exercise.

The application of microarray analysis and clustering in exercise physiology using human peripheral blood has been reported before by very few other authors. In contrast to our study with whole blood, Connolly et al. investigated peripheral blood mononuclear cells of 15 healthy individuals before and after 30 min of exercise, in addition to 60 min into the recovery period (3). Affymetrix microarrays were used and detected genes were clustered into genes related to stress response, inflammation and repair. Some, similarly exercise-regulated genes were detected in their study, and also in the present study: CD14, Chemokine ligand 4, Colony stimulating factor 3 receptor (CSF3R), HSPB1, IL-1 receptor type 2, IL-1receptor antagonist and IL-2 receptor beta. Alterations in gene expression likely depend on the type, duration and intensity of exercise. Furthermore, such changes probably vary between sampling times, between cell types and also between the different microarray platforms with their different number of investigated genes.

Another study by Sonna et al. analyzed pooled RNA from peripheral blood mononuclear cells of four male Marine recruits with symptoms of exertional heat injury (EHI) compared to healthy Marines at presentation in the hospital, 2-3 h after cooling, and 1-2 days later (23). Among these genes, they found sequences that are heat-shock responsive in peripheral blood mononuclear cells in vitro, a number of non-heat shock associated sequences, and several non-specific stress response genes. HSPB1 was consistently up-regulated in both of these studies (3,27) and also in the present study. Besides the influential factors mentioned

above, heterogeneous ethnic origin, environmental conditions, and the pooling of RNA may contribute to differences between the studies.

In contrast, Hilberg et al. analyzed whole blood cells using a spotted inflammation-centered oligonucleotide DNA microarray (9). They demonstrated gene expression changes in leukocytes 2 h and/or 6 h after exercise in six healthy individuals. Six clusters of similarly responding genes were identified. Consistent with the present findings and those of Connolly et al. (but not Sonna et al), two genes were similarly up-regulated: CSF3R and CD14.

Our data have shown that physical exertion is associated with a distinct pattern of gene expression. It is also likely that other patterns could be established for other physiological states and pathologies. A potential application of gene expression profiling using microarrays in exercise physiology has been shown recently for the chronic fatigue syndrome by Whistler et al. (29). Chronic fatigue syndrome is defined by debilitating fatigue that is exacerbated by physical or mental stress. The exercise response of patients with chronic fatigue syndrome was compared with that of healthy controls, and each group showed a distinct pattern of exercise-responsive genes.

Another example where a diagnostic gene expression profile would be of utility could be the overtraining syndrome. Overtraining syndrome is a condition that develops in athletes subjected to heavy training and/or other sources of stress in which their performance decreases and does not recover despite two weeks or more of relative rest (1; 22). The diagnosis of overtraining is usually complicated because there is a lack of universally agreed diagnostic criteria. Our findings might be a first step to develop a new diagnostic method. A gene expression fingerprint might define and characterize the overtraining syndrome and may therefore be a useful tool in early diagnosis of this condition. A very first approach in this direction was the gene expression profiling of neutrophils in Chinese Qigong practitioners (11). The microarray data reflect an enhanced immunity in the Qigong-trained compared with untrained subjects. Further studies with a representative number of athletes in various different states of training should be performed. This would allow assessment of the health and conditioning of athletes by analyzing peripheral blood.

Conclusion

We have investigated gene expression in peripheral blood of eight half marathon runners using a cDNA microarray focused on inflammatory markers. Applying microarray analysis we identified a gene expression fingerprint in humans both immediately following exercise and 24h after exercise, during the recovery phase. In general, gene expression profiling can help to characterize tissues or diseases, may give hints for special therapies and may serve in the future as a powerful research tool in exercise, as for example in diagnosing or preventing athletes from overtraining.

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