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Effect of exercise on gene expression profile in unfractionated peripheral blood leukocytes

Seiji Nakamura [a,*], Michie Kobayashi [a], Tomohiro Sugino [b], Osami Kajimoto [b], Ryo Matoba [a], Kenichi Matsubara [a]

[a] DNA Chip Research Inc., Suehirocho, Tsurumi-ku, Yokohama 230-0045, Japan
[b] Soiken Inc., Shinsenri-higashimachi, Toyonaka 560-0082, Japan

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ABSTRACT

A 4-h bout of exercise induces immunomodulatory effects. Peripheral blood was withdrawn before, and at 4, 8 and 24 h after the start of exercise. RNA from the unfractionated white blood cells was analyzed using Agilent human 44 K microarray. The expression profiles were sorted into seven clusters based on their unique time-dependent kinetics. In a separate experiment, cell-specific markers were collected and compared among the members in each cluster. Two clusters were assigned as representing neutrophils, one as NK cells, and another mostly as T cells. Three clusters seemed to be mixtures of several cell types. Extension of this approach to other systems is discussed.

Introduction

Exercise, training, and fatigue induce immunomodulatory effects, such as changes in the number and properties of neutrophils, B, T and NK cells, and monocytes in the peripheral blood. Because of the importance in medical and sports sciences, active studies have been made with white blood cells in the peripheral blood (for a review, see [1]). The recent introduction of microarrays has promoted extensive research activities especially on white blood cells, such as studies on cytokines, receptors, apoptosis, and control of cell proliferation [1–7]. However, almost all the microarray studies have been done with so-called peripheral blood mononuclear cells (PBMC). These cells, which are obtained by banding in a density gradient, represent only a fraction of the white blood cells in whole blood. Global studies using whole immune cells are expected to provide more important information. In addition, a significant fraction of these early microarray works have relied upon in-house-made cDNA microarrays, that has made it difficult to comparatively analyze the studies of genes among different investigators.

We report here, the results of our analyses on exercise-dependent transcription profiles using whole blood cells. We employed Agilent human 44 k microarrays that can cover the entire human genome.

Materials and methods

Participants and exercise protocol. Healthy, physically active male subjects (n = 5, 44.2 ± 9.4 yr), who had not been involved in any kind of resistance or endurance exercises, were assigned to a stationary bicycle at 80% of their predicted maximum workload in a room where temperature and humidity were kept at 25 ± 1 °C and 50 ± 10%, respectively. They refrained from eating food and drinking alcohol, coffee, or tea for 12 h before the exercise, and were asked to report to the laboratory at 8:30 am. The subjects were seated for 30 min, blood (2.5 ml) was collected by venous puncture for the time zero sample, and then they started exercise on an electrically braked cycle ergometer with rest at every 1 h. After 4 h of exercise, they were asked to take some food and rest for 4 h. They were asked to report to the laboratory at 24 h after start of the exercise. Blood (2.5 ml) was collected by venous puncture at 4, 8 and 24 h after the start of exercise. Blood was also collected for cell counts and biochemical markers. This study adhered to human experimentation guidelines of the Helsinki Declaration. The protocol was approved by the Ethics Committee of Soiken Inc. and Soiken Clinic. All participants were volunteers who gave informed consent.

Blood sampling and analyses. Blood (2.5 ml) was collected into PAXgene Blood RNA Kit (QIAGEN, Valencia, CA) [8], kept at room temperature for 2–4 h, followed by cooling. Total RNA was extracted according to the manufacturer's protocol, assayed for RNA, and signal intensity of the labeled cRNA measured by hybrid-

* Corresponding author. Fax: +81 45 500 5219.
E-mail address: nakamura@dna-chip.co.jp (S. Nakamura).

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ization to Agilent 44 k human genes microarray (Agilent Technologies, Santa Clara, CA). No protocols for elimination of hemoglobin mRNA were adopted (see Discussion and Supplementary comment). The raw microarray data are deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO Series GSE18966).

RNA from mononuclear (MON) cells and polymorphonuclear (PMN) cells. Independent of the above studies, we took blood from two age-matched male subjects, who did not participate in the exercise. The blood was collected into EDTA-containing buffer, and immediately separated into MON cells and PMN cells in a gradient centrifugation in Ficoll–Hypaque according to the manufacturer's protocol (DS Pharma Biochemical, Osaka, Japan). RNA was prepared with RNeasy Mini Kit (QIAGEN), analyzed by Agilent microarrays, and gene expression profiles in MON cells and PMN cells were compared for cell-specific markers. Since the RNA from two donors behaved identically, the two data were combined. Genes that were expressed in MON cells twice or more strongly than in PMN cells were defined as mononuclear-cell-markers (4858 genes), and genes that were expressed in PMN cells twice or more strongly than in MON cells were defined as polynuclear-cell-markers (4983 genes).

Cell-specific gene extraction using GEO microarray data. To further obtain detailed blood cell markers, we analyzed GSE3982 [9], sets of blood cell microarray data in NCBI GEO. The criteria of cell marker extraction were similar to that of the extraction of polynuclear-cell-markers and mononuclear-cell-markers noted above.

Data analyses. The microarray intensity values were processed by quantile method using the GeneSpring GX10 (Agilent Technologies) so as to normalize the range of expression intensities for inter-microarray. Gene level data were normalized against the time zero value. Only those genes (23,167) whose expression data were available in all of the 20 hybridization microarrays (viz. 5 persons $\times$ 4 time points) were taken for further analyses. The cclust package under the R environment (http://cran.at.r-project.org) was used to conduct K-means clustering for maximum separation.

Results

Participants

Five subjects participated in the exercise, donated blood at 0, 4, 8 and 24 h. RNA was prepared as in Materials and methods section. The anthropometric and physiological characteristics appear in Supplementary Table 1. The blood biochemical data before and after the exercise are shown in Supplementary Table 2.

Leukocyte counts

Exercise invoked changes in leukocyte counts (Table 1A), among which the abrupt rise in number (ca. $1.5 \sim 2x$) of neutrophils at 4 h was the most prominent. This finding is in accord with the foregoing reports [3,10,11]. By 8 h, the number reached a plateau. At 24 h, they returned completely to the original level. Lymphocyte counts were less dramatic. Other cells, such as eosinophils, basophils, monocytes were also counted, but since the counts were small, further studies were not done in this work. Leukocyte subpopulations (%) were less dramatic than leukocyte counts (Table 1B).

Analyses of expression profiles

Expression profiles of 23,167 genes were obtained with each of the participants. The expression data were clustered using Davies–Bouldin Validity Index [12] for maximum cluster separation. Seven clusters were separated as shown in Fig. 1.

Clusters 1 and 2 consisted of genes that rose abruptly upon exercise, levelled off at 8 h, and returned to the original level at 24 h, the behavior was quite similar to that of neutrophil cell counts (Table 1A). When we compared the 617 genes in cluster 1 with the mononuclear- and polynuclear-markers (Table 2), 455 matched to polynuclear-markers (p-value < E-11). Cell markers extracted from GEO blood cell microarray data showed that the majority of these genes are from neutrophils (p-value = 1.89E-10). Some representative genes are Ras-related C3 botulinum toxin substrate 2 (RAC2) [13,14], phosphatidylinositol 3,4,5-triphosphate-dependent RAC exchanger 1 (PREX1) [14,15], both of which regulate neutrophil NADPH oxidase connected to genesis of reactive oxygen species (ROS) for killing the invading microorganisms. Thus, we conclude that the cluster 1 genes are, or at least a significant fraction of them are, neutrophil markers. Similarly, the majority of the RNA in cluster 2 represent neutrophil RNA. Here, there are genes characteristic to the neutrophil, such as neutrophil cytosolic factor 1 (NCF1) [16,17], NCF4 [18], oncostatin M (OSM) [19,20], matrix metallopeptidase 25 (MMP25) [21], arachidonate 5-lipoxygenase (ALOX5) [22]. The levels of the changes of genes in these two clusters were more like that of the neutrophil counts than the neutrophil subpopulation (%) (Table 1A and B). That the clusters 1 and 2 differ may represent heterogeneity of the neutrophil population. We were not able to identify the major component RNA in cluster 3.

Cluster 4 whose expression level dropped significantly at 8 h, represents NK cells. Evidence for this comes from the high density of mononuclear-markers in this cluster (Table 2, p-value = 3.04E-11), and the NK cell-specific markers from GEO data (p-value = 1.89E-8).

Table 1
Exercise-induced changes in leukocyte count (A) and subpopulation (B).

	0 h (before exercise)	4 h (after exercise)	8 h (4 h after exercise)	24 h (20 h after exercise)
(A)				
WBCs (cells/μl)	4760 ± 1078	11,440 ± 2659**	10,100 ± 1748**	5120 ± 680
Neutrophils (cells/μl)	3245 ± 645	8597 ± 2916*	8009 ± 2186**	2905 ± 360
Lymphocytes (cells/μl)	1056 ± 352	2092 ± 871	1503 ± 446	1729 ± 260*
Monocytes (cells/μl)	295 ± 91	558 ± 222	453 ± 90*	311 ± 54
Eosinophils (cells/μl)	143 ± 93	127 ± 100	76 ± 47	137 ± 112
Basophils (cells/μl)	22 ± 12	66 ± 87	59 ± 46	37 ± 32
(B)				
Neutrophils (%)	68.6 ± 3.4	74.3 ± 8.9	78.3 ± 7.2*	56.9 ± 3.3**
Lymphocytes (%)	21.9 ± 3.5	18.6 ± 6.9	15.7 ± 6.2	33.8 ± 2.8**
Monocytes (%)	6.2 ± 1.6	5.3 ± 2.6	4.6 ± 1.3	6.1 ± 0.9
Eosinophils (%)	2.9 ± 1.4	1.2 ± 0.8	0.8 ± 0.5*	2.6 ± 1.7
Basophils (%)	0.4 ± 0.2	0.7 ± 0.9	0.6 ± 0.4	0.7 ± 0.5

Values are means ± SD. WBCs, white blood cells.
* $p < 0.05$ against time zero value.
** $p < 0.01$ against time zero value.

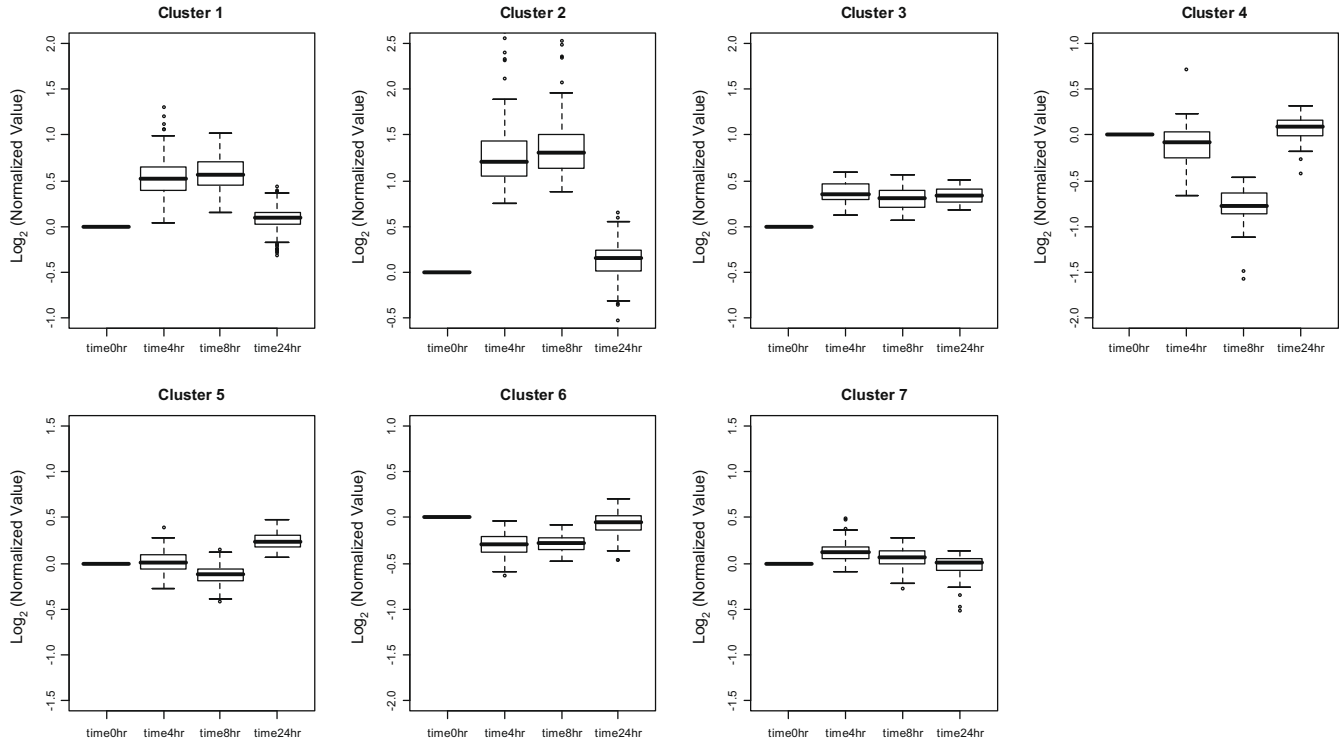

Fig. 1. Boxplots with seven gene clusters. Expression profiles of 23,167 genes were obtained on each participant. Data obtained on five participants and 4 time points were assembled into a matrix. Under the R software environment using the cclust package, maximum cluster separation was achieved by the K-means method, applying Davies–Bouldin Validity Index [12]. Eleven clusters were thus obtained. Only those genes (3478) that belonged to the same cluster with at least four out of five participants were saved. As the exercise started in the morning and ended in the afternoon, genes that are subjected to day-rhythm (Nakamura, personal communication) were further eliminated. Finally, 1411 genes in seven clusters were extracted for the boxplots. Each plot indicates the median value of five individuals of each gene. The bottom end of the box, the line inside the box, and the upper end of the box indicate, respectively, 25, 50 and 75 percentile of the plots. The upper (bottom) end of the whisker indicate the maximum (minimum) value within 1.5 times the length of the box. The scale of the vertical axes is binary logarithm.

A representative gene is granulysin (GNLY) which is an antimicrobial protein produced by human cytolytic T lymphocytes and natural killer cells [23]. The NK expression profile is unique in that expression drops significantly at the late stage of the exercise protocol (8 h). This finding is in accord with the previous reports that the NK cell number decreases after exercise, and recovers by 24 h [24,25].

Cluster 5 consists mostly of lymphocytes, because the mononuclear-markers are predominant (Table 2). Cells in this cluster are dominated by T cells, although there are markers from other cells. Cluster 6 also consists of mononuclear cells, but this cluster apparently consisted of a mixture of different cells. We could not assign cell-specific marker genes to cluster 7.

Inter-individual variations

Inter-individual variations were also examined. Fig. 2 shows the time-dependent expression of some of the genes among the five individuals. The results clearly showed that the genes we are dealing with are not subject to inter-individual variations.

Discussion

Analyses of whole blood cell behavior using expression profiles of genes

Exercise induces dramatic transient physiological changes in the body, including elevated blood flow, elevated body temperature, off-balance of hormones, appearance of heat-shock protein, oxidative stress, production of interleukins and receptors as well as many other substances related to inflammatory processes. Some of these physiological effectors have been discussed along with their effects on blood immune cell system, the so-called effect on immunocompetence. Among the changes in immunocompetence, the abrupt rise in neutrophil cell number is most prominent [10,11,26]. Other cells, such as NK cells, monocytes and their subclass members are also affected [27].

We approached this problem using gene expression profiles of whole peripheral white blood cell RNA. We were able to show, by assigning the time-dependent expression profiles into unique clusters, and then identify the clusters using cell-specific marker RNA collections that has been obtained by independent methods. The results were in good agreement with the cell-counting data: abrupt rise in neutrophils, the significant drop of NK cells at late stages of the exercise, the relatively flat time course profile of T cells (Fig. 1 and Table 1). This approach provides not only an entirely new way to follow cell behavior, but also provides other information, such as whether there might be different gene expression control within a group of cells.

Although it might be premature at this stage, the two neutrophil clusters, clusters 1 and 2, could be representing different neutrophil populations. Further study is needed to clarify this point.

The rapid rise in neutrophils is called demargination [28] and has been discussed with causative agents [11,27,29,30], and with adhesive properties of granulocytes [29,31–33]. Most of these discussions have been based on findings obtained with cultured cells. The proposal by Fisher [34] is simple and attractive, in that release of IL-6 from activated uninjured muscles would play the major role for the demargination. We argue that in the presence of such complex physiological effectors, working with whole blood using

Table 2
Matching cell-specific markers with each exercise-cluster genes.

Cluster No.	Cluster genes	Cell marker	Cell marker genes	Matched genes	*p*-Value
1	617	2fold_polynuclear_cell_markers	4983	455	<E-11
1	617	2fold_Neutrophil_markers	712	92	1.89E-10
1	617	2fold_Eosinophil_markers	256	10	0.005696
1	617	2fold_Basophil_markers	258	8	0.042251
1	617	2fold_Macrophage_markers	243	6	0.160809
1	617	2fold_Th2_cell_markers	58	2	0.216586
1	617	2fold_Th1_cell_markers	61	2	0.233194
1	617	2fold_Immature_dendritic_cell_markers	157	1	0.907507
1	617	2fold_mononuclear_cell_markers	4858	6	1
2	105	2fold_polynuclear_cell_markers	4983	100	<E-11
2	105	2fold_Neutrophil_markers	712	42	4.92E-11
2	105	2fold_B_cell_markers	218	1	0.428463
3	38	2fold_polynuclear_cell_markers	4983	9	0.034823
3	38	2fold_mononuclear_cell_markers	4858	8	0.073005
4	36	2fold_mononuclear_cell_markers	4858	21	3.04E-11
4	36	2fold_NK_cell_markers	93	5	1.89E-08
4	36	2fold_Th1_cell_markers	61	1	0.052115
4	36	2fold_Eosinophil_markers	256	1	0.201605
5	231	2fold_mononuclear_cell_markers	4858	85	<E-11
5	231	2fold_Central_memory_T_cell_markers	47	4	0.000144
5	231	2fold_B_cell_markers	218	2	0.347157
5	231	2fold_Eosinophil_markers	256	1	0.765002
5	231	2fold_Basophil_markers	258	1	0.767654
6	225	2fold_mononuclear_cell_markers	4858	53	6.32E-07
6	225	2fold_Immature_dendritic_cell_markers	157	2	0.212657
6	225	2fold_B_cell_markers	218	2	0.335602
6	225	2fold_Macrophage_markers	243	1	0.737793
6	225	2fold_Basophil_markers	258	1	0.758652
6	225	2fold_polynuclear_cell_markers	4983	23	0.836826
7	159	2fold_polynuclear_cell_markers	4983	26	0.069524
7	159	2fold_B_cell_markers	218	1	0.571597
7	159	2fold_Macrophage_markers	243	1	0.611396
7	159	2fold_Eosinophil_markers	256	1	0.630616
7	159	2fold_Neutrophil_markers	712	2	0.764587
7	159	2fold_mononuclear_cell_markers	4858	6	0.999911

Using GeneSpring GX10 "Find Similar Entity Lists" function, we assessed overlap between each exercise-cluster genes and each cell-specific marker genes. In the "Find Similar Entity Lists" function, the *p*-value is calculated using hypergeometric distribution.

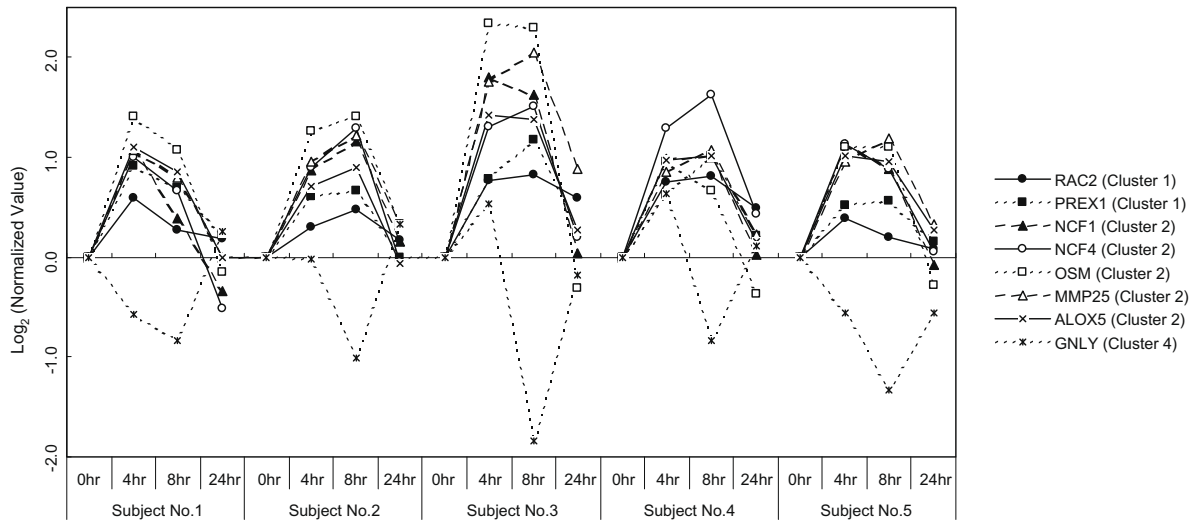

Fig. 2. Intensity of some of the cluster genes among the five individuals. Intensity of some of the representative genes in the clusters are shown. The scale of the vertical axes is binary logarithm. RAC2, Ras-related C3 botulinum toxin substrate 2. PREX1, phosphatidylinositol 3,4,5-triphosphate-dependent RAC exchanger 1. NCF1, neutrophil cytosolic factor 1. NCF4, neutrophil cytosolic factor 4. OSM, oncostatin M. MMP25, matrix metallopeptidase 25. ALOX5, arachidonate 5-lipoxygenase. GNLY, granulysin.

experimental animals, sampling at short intervals, and under various experimental conditions will provide a short cut to the answer.

The roles played by the demarginated neutrophils in the absence of muscle damage is another interesting question, and altered phagocytic, cell-killing, oxygen burst activities have been reported [3]. We attempted to compare the neutrophil RNA before and after exercise by isolating the cells in density gradients. We observed, however, little recovery in the neutrophil RNA after 4 h of exercise, suggesting instability. Radom-Aizik [3] performed a similar experiment and suggested a difference. Further work is

necessary. In this connection, it might be interesting to point out that neutrophils were clustered in our experiment, into 1 and 2, suggesting that the cells are heterogeneous.

Transcripts in NK cells showed a characteristic time course, i.e. there was a sharp drop in number several hours after the start of the exercise, followed by recovery on the next day (Fig. 1, cluster 4). The decline and recovery of RNA is in accord with the foregoing findings from cell counts [25]. To our knowledge, this is the first finding from expression analyses that genes in NK cells behave in an exercise-dependent fashion.

Some workers [24,25] reported that the NK cell number rises at an early stage of exercise. The difference between these reports and our findings may well be due to a difference in exercise condition, because heat-shock protein in the blood affects the NK cells [24]. Critical evaluation of the condition of the exercise may be needed in future studies, and here, again, whole blood analyses will provide a convenient platform.

Genes in cluster 5, likely to be representing the T cells, behaved uniquely. However, because of the relatively flat profile, we were unable to compare it with data on cell counts. We met similar problems with genes in clusters 6 and 7.

Inter-individual variations and the concordant genes

Fig. 2 shows that our data specify characteristic exercise-dependent gene expression signatures, free from inter-individual differences. This precaution is important when we extend this approach to other problems, such as stress, endurance training, food intake and ageing.

There have been several reports describing exercise-associated unique gene expression [4,7]. The concordant genes may be accounted for as exercise-specific and may be used as biomarkers for monitoring the effect of exercise, formulating post-training protocols, or testing adequacy of supplements.

The problems in whole blood analyses

Measurement using whole blood is the closest we can get to the in vivo condition. However, there are two problems associated with the simple protocol presented here: (i) Possible interference or distortion of microarray assay data by hemoglobin RNA. (ii) Expression data from unfractionated cells is too complex for subsequent analyses.

Problem (i) has yet to be solved completely. Methodological or technical insufficiencies will bring about inconsistent or false results. However, in practice, results obtained with the simple protocol as adopted here are not seriously distorted, yielding reproducible results. Clearer results may be available by modified protocols, but such elaborate protocols bring about other problems. These topics are treated in the supplement. Above all, the simple protocol is advantageous for comparison of data: results may be studied side-by-side, irrespective of whether they are obtained from multicenters, from long-run sample collections on a large scale, or from analyses by different laboratory staff. We feel that it is reasonable, although perhaps not ideal, to follow this simple study design, until a superior simple method appears.

Problem (ii) stems from the fact that many of the observable expression changes are related to cell population shifts and gene expression changes. By analyzing data from multiple samples (time course kinetics and clustered genes), and by comparing the clustered genes with a set of genes collected from a different source (the density-banded MON cells and PMN cells and the GEO gene set from fractionated cells), we could assign the genes identified from their behavior to known cells. This approach helped us elucidate the behavior of neutrophils, NK cells and T cells. This method may well be extended to other topics, such as age, stress, and food or drug intake, to examine the behavior of each cell under the influence of stress. Improvement in number and quality of the cell-specific marker collection, as well as characterizing the uniquely acting genes should promote future progress.

Acknowledgments

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Appendix A. Supplementary data

IT contains the anthropometric and physiological characteristics (Supplementary Table 1), the blood biochemical data before and after the exercise (Supplementary Table 2) and Supplementary comment about elimination of hemoglobin mRNA. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.150.

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