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Gene Expression Profile of Zinc-Deficient, Homocysteine-Treated Endothelial Cells

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Abstract

In the post-genome period, the technique for identifying gene expression has been progressed to high throughput screening. In the field of molecular nutrition, the use of screening techniques to clarify molecular function of specific nutrients would be very advantageous. In this study, we have evaluated Zn-regulated gene expression in Zn-deficient, homocystein-treated EA.hy926 cells, using cDNA microarray, which can be used to screen the expression of many genes simultaneously. The information obtained can be used for preliminary assessment of molecular and signaling events modulated by Zn under pro-atherogenic conditions. EA.hy926 cells derived from human umbilical vein endothelial cells were cultured in Zn-adequate (control, 15 μM Zn) or Zn-deficient (experimental, 0 μM Zn) Dulbecco's MEM media under high homocysteine level (100 μM) for 3 days of post-confluency. Cells were harvested and RNA was extracted. Total RNA was reverse-transcribed and the synthesized cDNA was labeled with Cy3 or Cy5. Fluorescent labeled cDNA probe was applied to microarray slides for hybridization, and the slide was then scanned using a fluorescence scanner. The expression of seven genes was found to be significantly decreased, and one significantly increased, in response to treatment of EA.hy926 cells with Zn-deficient medium, compared with Zn-supplemented medium. The upregulated genes were oncogenes and tumor suppressor genes, cell cycle-related genes and transporter genes. The down-regulated gene was RelB, a component of the NF-kappaB complex of transcription factors. The results of this study imply the effectiveness of cDNA microarray for expression profiling of a singly nutrient deficiency, namely Zn. Furthur study, using tailored-cDNA array and vascular endothelial cell lines, would be beneficial to clarify the molecular function of Zn in atherosclerosis, more in detail.

Key words: Zn-deficiency, endothelial cells, EA.hy926 cell, homocysteine, cDNA microarray, atherosclerosis

INTRODUCTION

The physiology and biochemistry of Zn as an essential nutrient has been appreciated for over 50 years (1). However, the more recent discovery that Zn is an essential structural component in over 2000 Zn finger proteins, many of which are transcription factors (2), has demonstrated the importance of Zn nutrition at a fundamental molecular level. Acute Zn deficiency has profound effects at the whole organism level, including fetal teratogenesis, impaired growth and immunity and skin lesions (3). These effects can be directly related to molecular events, such as the suppression of thymidine kinase gene expression (4), which inhibits cell division and therefore adversely affects growth and tissue repair. Elucidation of Zn effects at a molecular level has previously required a meticulous gene-by-gene analysis of pathways suspected of being affected under physiological conditions.

The development of rapid screening techniques such as microarray and 2D-gel electrophoresis has opened up the possibility for screening large numbers of genes or proteins that might be Zn regulated. There are a number of potential limitations and pitfalls of these techniques (5), but when applied prudently and with appropriate statistical analysis (6), they are very powerful tools to accelerate discovery of nutrient-affected molecular pathways. They also give a better understanding of individual nutrient effects in integrated physiological systems, particularly where subtle effects of chronic deficiency may not have a marked effect on the expression of any particular gene or protein. In the case of chronic or marginal Zn deficiency, effects such as suppressed growth and immunity, are not pronounced but can be detected in careful controlled studies (1). There is no reliable diagnostic indicator of Zn status, but it is estimated that a third of the World's population is Zn deficient (7), including marginal deficiency in developed countries.

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While protection by adequate Zn intake against vector-based diseases has been appreciated for some time, a protective role against heart disease (8) and cancer (9) has only recently been discussed. A role for Zn in protecting against atherogenesis has been proposed (10,11), suggesting that there may be several mechanisms by which Zn can be effective in protecting the vascular endothelium from damage by pro-oxidant nutritionally-related compounds, such as linoleic acid (12). Endothelial cell damage attracts recruitment and adhesion of circulating monocytes, which are consequently activated to become macrophages and transmigrate though the damaged endothelium (13). In the presence of oxidized LDL, the intimal macrophages become foam cells and an inflammatory response causes cytokine-induced endothelial apoptosis and smooth muscle cell proliferation. Such reactions can ultimately result in the formation of fibrous plaque which may occlude arteries, including the coronary artery, and result in ischemia and myocardial infarction.

Our research objective is to identify how physiological Zn deficiency affects the key signaling and metabolic pathways that are affected by stress factors which damage the endothelium. A major focus of the work is to elucidate the pathways affected by homocysteine and the impact of Zn deficiency on the harmful effects of homocysteine in endothelial cells. Homocysteine is a non-essential amino acid and product of methionine metabolism whose circulating levels increase in response to folic acid and vitamin B_{12} deficiency (14). Although homocysteine has reducing properties, it can initiate redox reactions with for example copper (15,16) which may generate free radical species. It also forms disulphide links with protein and amino acid cysteinal thiols. There is some controversy as to whether raised blood homocysteine levels are a cause or effect of coronary heart disease.

In the present study, we have used microarray technology to investigate the effect of Zn deficiency on gene expression in EA.hy926 cells exposed to 100 M homocysteine. The objective of this study was to identify Zn-regulated genes under conditions of homocysteine treatment.

MATERIALS AND METHODS

Zn-chelexed FBS containing media and Zn-treatment

Chelex-treated fetal bovine serum (Sigma) was used to limit Zn availability in composed media. Chelex-100 ion exchange resin (5 g; Bio-Rad) was added to fetal bovine serum (100 mL), and the mixture was stirred over night at 4°C. After removal of the resin, the solution was then filter-sterilized into polyethylene centrifuge tubes. Zn-depleted media was then prepared using Dulbecco's MEM (DMEM), which contained 25 mM HEPES, 45 mg/L glucose, and with pyridoxine, but without sodium pyruvate (Gibco), 100 mL/L chelexed FBS, 20 mL/L HAT supplement, and 100 u/mL penicillin, 100 g/mL streptomycin solution. All plastic ware used for media preparation and cell culture work was washed with Acationox detergent (Baxter Scientific Products, McGaw Park, IL) before use.

The media containing chelex-treated FBS was used for Zn-deficient treatment. For the Zn-adequate treatment, the media containing chelex-treated FBS was supplemented with a $ZnCl_2$ to a nominal level of 15 μM Zn.

Zn measurement in media components

Zn concentration in normal and chelexed-FBS was measured to confirm the depletion of Zn. Samples (1 mL) were dried at heating block at 107°C overnight and wet-digested. Samples were kept at room temperature with addition of 100 μL concentrated nitric acid (trace element free, Fluka). After then, the samples were heated upto 100°C to digest the samples and diluted with metal-free water to measure using atomic absorption spectrophotometer.

Experimental design and EA.hy926 cell culture

EA.hy926 cells were originally derived from human umbilical vein endothelial cells and therefore have the characteristics of vascular endothelial cells. The cells were maintained in DMEM growth media, which contained normal FBS, until they became confluent. Since EA.hy926 cells showed the expression of von Willebrand factor as a differentiation marker for endothelial cells after confluency, the cells were treated post-confluency. Fresh growth medium was exchanged every 3 days. After 3~4 days of confluency, the cells were exposed to 100 μM homocysteine, using either Zn-deficient media containing chelexed FBS (0 μM Zn, Zn-) or Zn-adequate media containing chelexed-FBS with added $ZnCl_2$ (15 μM Zn, Zn+) for 3 days after confluence. Stock $ZnCl_2$ solution (10 mM) and homocysteine solution (10 mM) were freshly prepared. Cells were collected by centrifugation for 5 min at 1,000×g at room temperature.

cDNA glass slide microarray

The cDNA slides used in the present study were oligonucleotide micorarrays (Atlas™ Glass Human Microarray, Clontech, USA) and the genes were detected by fluorescently labeled probe using a slide scanner (Gene TAC™LS IV, Genomic Solution). To minimize the experimental error, all of the reagents for microarray analysis were purchased from the same company from which

the slides were obtained.

Microarray experiments were performed as four studies to compare gene expression in the cells exposed to Zn-deficient and Zn-adequate media. Dye swapping for each treatment study on pairs of slides was employed to correct for differences in dye intensity for normalization.

RNA isolation and assessing purity: After 3 days of Zn treatment, the cells were harvested and total RNA was immediately extracted by addition of phenol:chloroform two times to obtain pure RNA (Atlas^{TM} Glass Total RNA isolation kit, Clontech, CA, USA). After precipitating RNA by adding isopropanol and drying the pellet after centrifugation, RNA samples were dissolved in RNase-free water at a concentration of 1~2 μg/μL and stored at -80°C. The purity of total RNA was verified by measuring the ratio of A260/A280. Pure RNA has a ratio of 1.9~2.1. Total RNA purity was also examined by separation on a 1.2% agarose/formaldehyde gel. Total RNA from mammalian sources should appear as two bands (28S and 18S ribosomal RNA) at approximately 4.5 and 1.9 kb. The ratio of intensities of the 28S and 18S rRNA bands should be 1.5~2.5:1. Lower ratios are indicative of degradation.

cDNA probe synthesis: cDNA from total RNA was synthesized according to the manufacturer's instructions (Atlas^{TM} glass fluorescent labeling kit, Clontech, CA, USA). Total RNA was reverse transcribed using a random primer mix and MMLV reverse transcriptase, and purified using 0.5 μL 0.5 M EDTA (pH 8.0) and 5 μL Quickclean resin by centrifuging at 14,000 rpm for 1 minute. The synthesized cDNA pellet was dissolved in fluorescent labeling buffer.

Fluorescent dye, Cy3 and Cy5 (FluroLink^{TM} Cy3 monofunctional dye, PA 23001, Amersham Pharmacia Biotech) was incorporated into the synthesized cDNA. The fluorescent dye coupled cDNA probe was washed with 70% ethanol or labeling buffer provided (Atlas^{TM} glass fluorescent labeling kit, Clontech, CA, USA) and stored in deionized H_2O in the dark at -20°C.

Hybridization and scanning: Following probe synthesis, the fluorescently labeled-probes were hybridized overnight to microarrays using the protocol supplied with the slides (Atlas^{TM} Glass Microarrays, Clontech, CA, USA). After washing the slides with 1X SSC solution, the dried slides were scanned using a fluorescence scanner (Gene TAC^{TM} LS IV, Genomic Solution). The microarray statistical analysis was performed with Scanalyze2 (www.microarrays.org/software.html).

RESULTS AND DISCUSSION

Zn level in chelexed FBS

The Zn level in Zn-adequate and deficient media was nominally 15 μM and 0 μM Zn, respectively. The measured Zn concentration of chelexed-FBS was 0.108 μM by atomic absorption spectroscopy. Cu and Fe concentrations in chelexed-FBS were not changed, thus chelexing only removed Zn in FBS.

Cell morphology

The cells appeared to be morphologically unaffected by the Zn-deficient treatment and were adherent to the plastic flasks and visually very similar to the Zn-adequate cells. Thus, until harvesting of the cells, Zn-deficiency didn't affect to the cell survival.

RNA purity

In each of the four studies, RNA yield and purity were assessed for both Zn-deficient and Zn-adequate treatments and the RNA quality was considered suitable for microarray work (Fig. 1). A260/ A280 ratios were within the range of 1.85~1.94 and the 28S rRNA and 18S rRNA intensity was considered appropriate for cDNA probe synthesis.

Slide normalisation

Cy3 labelling gave stronger signals than Cy5 on the microarray slides and dye swapping for each treatment on pairs of slides was employed to correct for differences in dye intensity. By scanning, the slides showed well defined spots and a range of intensities across the arrays. Care was taken to exclude any artifact fluorescent particles from the analysis. All spot intensities were $\log_2$ transformed and a lowess normalisation was performed for each array to remove intensity dependent channel effects. The plots of log intensity (Fig. 2) show that normalisation was successful in removing any unwanted curvature from the data. Correlation coefficients for normalized data were similar to those for non-normalised data (Table 1), with the exception of one slide (slide 3), which was therefore excluded from the analysis. Plots of markers and housekeeping genes were also made to

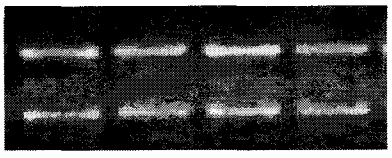

Fig. 1. 1.2% agarose gel separation of EAhy.926 cell RNA, showing 18S and 28S bands. RNA in lanes 1~2 are for Zn-deficient cells (0 μM) and RNA in lanes 3~4 are for Zn-adequate cells (15 μM).

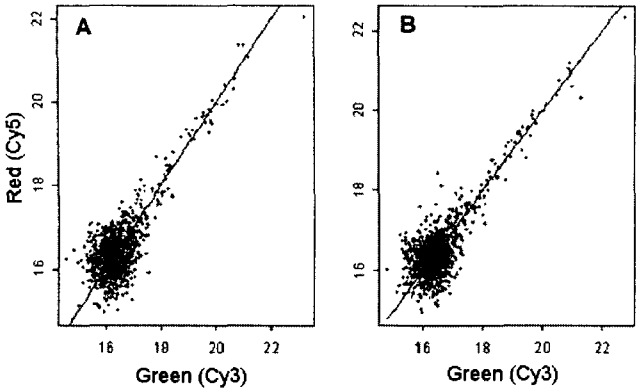

Fig. 2. Normalised plots of Zn-adequate (Cy5) versus Zn-deficient (Cy3) media treatment (A) and dye swap for the same treatments (B).

confirm that the relative Cy3 and Cy5 intensity of these genes for both treatments was similar (data not shown).

Gene expression profiles

Statistical analysis of the data demonstrated that the expression of 8 genes was significantly affected by treatment of EA.hy926 cells with Zn-deficient medium (Table 2). All except one gene were downregulated by exposure to this medium. The genes can be grouped on a functional basis into oncogenes and tumor suppressor genes, cell cycle-related genes, and transporter genes. The ErbB4 receptor is a human epidermal growth factor receptor and is most predominantly expressed in several breast carcinoma cell lines, in normal skeletal muscle, heart, pituitary, brain, and cerebellum (17) and in the vascular endothelium (18). There is as yet no evidence suggesting that this gene is Zn-regulated or that the ErbB4 receptor utilizes Zn for its function. Two p53-induced genes (PIG6 and PIG12) were downregulated by Zn-deficient medium treatment. Since p53 is a Zn-dependent protein (19), it is possible that reduced activity of this transcription factor could explain a down regulation in PIG expression. The PIG proteins have been predicted to be involved in radical oxygen species generation (20) which may induce apoptosis independently of other p53 related signaling pathways (21).

The two cell cycle related genes are not directly related to each other. The cholinesterase-related cell division controller (CHED) is a universal controller of the mitotic cell cycle (22). It includes the consensus ATP binding and phosphorylation domains characteristic of kinases and displays 34~42% identically aligned amino acid residues with other cdc2-related kinases. There is no published information concerning its relationship with

Table 1. Correlation coefficients for normalised and original intensity data

Study	Array	Dye-labeling (Swapping)	Cy5/Cy3 Correlation for original data (log-scale)	Cy5/Cy3 Correlation for normalised data (log-scale)
I	1	Zn+(Cy3)/Zn-(Cy5)	0.789	0.779
	2	Zn+(Cy5)/Zn-(Cy3)	0.895	0.897
II	3	Zn+(Cy3)/Zn-(Cy5)	0.290	0.524
	4	Zn+(Cy5)/Zn-(Cy3)	0.699	0.672
III	5	Zn+(Cy3)/Zn-(Cy5)	0.789	0.798
	6	Zn+(Cy5)/Zn-(Cy3)	0.814	0.819
IV	7	Zn+(Cy3)/Zn-(Cy5)	0.689	0.712
	8	Zn+(Cy5)/Zn-(Cy3)	0.809	0.793

Table 2. Genes significantly up (↑) or down (↓) regulated in response to treatment of EA.hy926 endothelial cells with zinc-deficient medium

Gene (GeneBank Accesstion No.)	Function		*P* value
erbB4 proto-oncogene; HER4; neuregulin receptor (L07868)	Oncogenes & Tumor Suppressors Intracellular Transducers, Effectors & Modulators	↓	0.04
PIG6 (AF010310 & AF010311)	Oncogenes & Tumor Suppressors	↓	0.05
PIG12 (AF010316)	Oncogenes & Tumor Suppressors Apoptosis-Associated Proteins	↓	0.04
CDC2-related protein kinase CHED (M80629)	Cell Cycle-Regulating Kinases Intracellular Kinase Network Members	↓	0.01
Cardiac muscle Na channel alpha subunit (M77235)	Voltage-Gated Ion Channels	↓	0.05
Sulfate transporter (U14528)	Facilitated Diffusion Proteins	↓	0.02
Transformation/transcription domain-associated protein (AF076974)	Other Intracellular Transducers, Effectors & Modulators Cell Cycle-Related Proteins	↑	0.03
Transcription factor relB;I-rel (M83221)	Transcription Activators & Repressors	↓	0.05

Zn. The other cell cycle protein, the transformation-transactivation domain-associated protein (TRRAP), is required for several Myc-mediated activities (23). The endothelial cells used in the present study show contact inhibition of cell division at confluence, and therefore it is not clear why these genes are affected by Zn deficiency. RelB is a subunit of the key transcription factor NF kappaB, which regulates the expression of many genes and signalling pathways related to apoptosis and the immune and inflammatory responses (24). However, RelB shows tissue specific expression and is best known for its regulatory role in dendritic cells (25). This subunit is not known to be affected by Zn, but NF kappaB activity is strongly inhibited by Zn in endothelial cells (26).

In conclusion, we have demonstrated the application of microarray technology to the study of Zn deficiency in an endothelial cell line. Eight genes were shown to be affected by exposure of EA.hy926 cells to Zn-deficient medium, seven of which, including oncogenes and tumor suppressor genes, cell cycle-related genes, and transporter genes, were down-regulated. RelB, a subunit of the key transcription factor NF kappaB, was upregulated. Clearly, these results require verification using an independent method of measuring gene expression, such as real-time PCR, and this work is in progress.

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