EXPERIMENTAL STUDY

Transcriptional Analysis of Endothelial Cell Alternation Induced by Atrial Natriuretic Polypeptide in Human Umbilical Vein Endothelial Cells

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Summary

The aim of this study was to explore how atrial natriuretic polypeptide (ANP) affects the properties and function of endothelial cells. Gene expression data GSE56976 generated at 0, 1, and 6 hours after ANP incubation in human umbilical vein endothelial cells (HUVEC) was used. Microarray data were preprocessed for differentially expressed genes (DEGs) in each time-dependent group. Next, gene ontology (GO), pathway analysis, and transcriptional regulation were performed. Co-expression clustering analysis of DEGs and functional enrichment analysis of co-expression modules were processed. RT-PCR analysis was performed to validate gene expression. DEGs were obtained and their counts were increased from 0 hours to 6 hours. No overlapping DEGs were obtained among the 3 groups. The DEGs of ANP_6hours, including TGFB2 (transforming growth factor, beta 2), LTF (lactotransferrin/lactoferrin), and ETV7 (Et s variant 7) were mainly related with cell apoptosis and immune responses. The DEGs in the network of ANP_0hour were mainly associated with epithelial ion transport processes. In addition, 3 co-expressed modules were detected. CSF2 (colony stimulating factor 2) and PF4 (platelet factor 4) of the blue module were related with cytolysis, while FXYD1 (FXYD domain containing ion transport regulator 1) and TGFB2 of the yellow module were mainly enriched in ion transport and the ovulation cycle. The expression of TGFB2 obtained by microarray analysis was consistent with that of RT-PCR. Ion transport could be affected promptly after ANP treatment, and subsequently, the cytolysis of vein endothelial cells may be promoted and endothelial permeability would be enhanced, followed by activated immune responses.

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Key words: Gene ontology, Differentially expressed genes, Co-expression clustering analysis, Functional enrichment analysis

Atrial natriuretic peptide (ANP) is a 28-amino-acid peptide hormone of cardiac origin.1 It has demonstrated a decisive action in endocrine mechanisms of activation and haemodynamic homeostasis and adjustments by its diuretic, natriuretic, and vasorelaxant effects, and it has a role in cardiovascular remodeling.2,3 Cannone, et al. suggested that ANP stimulates endothelial macromolecule/vascular permeability, and leaky endothelial cells as well-known cardiovascular risk factors are involved in the development of diseases such as atherosclerosis.4 More interestingly, ANP was found to prevent early relapse in cancers by preventing metastasis.5 The systemic inflammatory response which promotes cancer cell adhesion to vascular endothelial cells would be inhibited by ANP to prevent cancer metastasis.5 Since cancer cell attachment to endothelial cells is the initial step in metastasis, exploring the mechanism underlying ANP mediated inhibition of tumor cell attachment to vascular endothelial cells and the antimitastatic activities would help to prevent cancer recurrence after surgery. Nojiri, et al. have reported that the expression of E-selectin, a critical component in hematogenous metastasis, was suppressed by ANP in human umbilical vein endothelial cells (HUVEC).6 Serafino, et al. reported that ANP regulates cellular acidification and the Wnt/β-catenin signaling cascade to produce anti-proliferative effects in colorectal cancer cells.7 Therefore, ANP could be used in clinical practice for cancer therapy. However, further studies are needed to elucidate the detailed mechanism of the effects of ANP on cancer metastasis.

Primary endothelial cells (HUVEC) are extracted from the endothelium of veins from human neonatal um-
bilateral cords. They are usually used as a model system for the exploration of regulation in endothelial cells and their role in diseases. Whole-genome-wide transcript profiling is widely used to investigate the deregulated molecular mechanisms underlying complex diseases. In our study, HUVEC were treated with ANP, and the changes in gene expression level were evaluated in publicly available microarray datasets. Candidate genes that are closely connected with ANP-treatment were identified and functionally analyzed. The findings of this study may shed light on the underlying molecular mechanisms of ANP-related endothelial cell metastasis, and provide essential information with which to develop novel therapeutic strategies for the treatment of metastatic cancer.

Methods

Gene expression profile data: To explore the molecular mechanisms of ANP in vascular endothelial cells, the gene expression data GSE56976 for HUVEC was downloaded from NCBI GEO (Gene Expression Omnibus; http://www.ncbi.nlm.nih.gov/geo) as TXT files. This dataset was composed of data measured at 0, 1, and 6 hours after the induction of ANP (0.1 μM), and controls in 4 independent experiments. The platform for this data was GPL6480 Agilent-014850 Whole Human Genome Microarray 4x44K G4112F (Probe Name version) (Affymetrix Inc., Santa Clara, CA, USA).

Microarray data preprocessing: Gene expression matrix (Downloaded on 10/27/2015) was preprocessed using the Bioconductor preprocessCore package (Fred Hutchinson Cancer Research Center, Seattle, WA, USA) in R. Annotation files of the chip annotation file were downloaded and used to convert the probe IDs to the official gene symbol. If multiple probes were mapped to a single gene, the mean expression value was calculated, while individual probes mapped to more than one gene were removed.

Differential expression analysis: A comparative study of the experimental and control groups at each time point (ANP_0hour versus Control_0hour, ANP_1hour versus Control_1hour, and ANP_6hours versus Control_6hours) was performed. Expression differences between each comparison were analyzed using the unpaired Student t test in the Bioconductor limma package. DEGs (differentially expressed genes) with a P value < 0.05 and FC (fold change) ≥ 1.366 (llog 2 FC ≥ 0.45) were selected. In addition, gene expression clustering in each comparative group was conducted using the ggplot R Package.

Gene ontology (GO) and pathway analysis of DEGs: The frequently-used tool database for annotation, visualization, and integrated discovery (DAVID) is an open, online accessible application designed for comprehensive functional analysis of genes. We used it for gene classifications in GO and pathway-based analysis in our study (count ≥ 2 and P < 0.05). The Kyoto Encyclopedia of Genes and Genomes (KEGG) database that collects molecular networks for all organisms was used to obtain information for pathway enrichment analysis.

Transcriptional regulatory analysis of DEGs: Literature mining revealed known regulatory relationships between the transcription factors and their target genes. Genomatix software suite v3.4 (http://www.genomatix.de/cgi-bin/GeP S/gene-tf_analysis.pl?) (Munich, Germany) was used to identify candidate TFs in the DEGs list, following which the transcriptional regulatory network was constructed.

Co-expression module analysis: The 3 separate DEG lists of ANP_0hour, ANP_1hour, and ANP_6hours compared to their own controls were merged, and then the overall gene expression trends were analyzed to define co-expressed genes. WGCNA, an R package for weighted correlation network analysis, is available for phenotypic-correlated gene clusters (modules) and feature genes in each module. In this study, the WGCNA package was used to identify phenotypic modules that were significantly enriched by DEGs. The greater the absolute value of the correlation coefficient, the more consistent the expression trends of the genes, and the stronger the relationship of gene expression to phenotype.

Functional enrichment analysis of modules: Modular enrichment analysis for phenotypic modules obtained above was performed using the DAVID tool to identify significantly enriched GO-BP categories. The criteria for this analysis were a count ≥ 2 and P < 0.05.

RT-PCR validation of gene expression: HUVEC line cells (CBR130511) purchased from Cellbio Biotech Company (Shanghai, China) were cultured in high-glucose DMEM containing 10% FBS and 1% penicillin/streptomycin at 37°C in 5% CO₂. HUVEC in good condition were selected and seeded onto the 6-well plates at a density of 1.5×10⁵/mL for 12 hours. The cells were then incubated with 0.1 μM ANP (4135-s, Peptide Institute, Osaka, Japan) (dissolved in physiological saline solution) for 1 hour and 6 hours. Cells treated with equivalent amounts of physiological saline were used as controls.

The HUVEC were collected by centrifugation and total RNA was extracted with RNAiso Plus (9109, Takara Bio, Inc., Dalian, China) and reverse transcribed into cDNA according to the manufacturer’s protocol (RNA PCR kit, Takara). Finally, the mRNA expression levels of DEGs and GAPDH (loading control) were verified by real-time quantitative RT-PCR using an SYBR Green PCR master mix kit (4367659, Applied Biosystems, Foster City, USA).

Results

Data normalization: Microarray data before (Supplemental Figure 1A) and after (Supplemental Figure 1B) normalization are shown in the boxplot. The lines in the middle of the box representing the medians of all samples were all at the same levels. The normalized data were applicable for subsequent analysis.

DEGs in 3 comparison groups: The DEG counts obtained in 3 independent comparison groups are shown in Table I. The gene counts increased, in turn, with an increase in the time of ANP treatment. The intersection results of genes in the 3 comparison groups are displayed in Supplemental Figure 2A. No overlapping DEGs were obtained among the 3 groups. The hierarchically clustered heat maps of comparison of 0hour, 1hour, and 6hours are shown in Supplemental Figure 2B, C and D, respectively.

GO-BP terms and KEGG pathways of DEGs: Func-
tional enrichment analysis results of DEGs in ANP_0hour, ANP_1hour, and ANP_6hours are shown in Table II. Down-regulated DEGs of ANP_0hour were enriched in BP terms of ion transport and secretion, but the up-regulated DEGs were not. GO-BP categories including response to drug, stem cell maintenance, development and differentiation (up-regulated), muscle organ development, cellular amino acid derivative metabolic process, and oxygen and reactive oxygen species metabolic process were identified by DEGs of ANP_1hour. Those of ANP_6hours were enriched in BP terms of regulation of lipase activity (up-regulated), leukocyte tethering or rolling and cellular extravasation, and defense response to bacterium (down-regulated).

No pathways were enriched by DEGs of ANP_0hour or ANP_1hour. Those of ANP_6hours were related with hsa04080: Neuroactive ligand-receptor interaction, hsa04060: Cytokine-cytokine receptor interaction (up-regulated), hsa04640: Hematopoietic cell lineage and hsa04514: Cell adhesion molecules (CAMS) (down-regulated).

**TFs and transcriptional regulation:** Total 7 TFs including ZIC1 (Zic family member 1) and TP73 (tumor protein P73) (up-regulated), PAX1 (paired box 1) and EN1 (en-grailed homeobox1) (down-regulated) were identified in ANP_0hour. Another 7 TFs including TCF7L2 (transcription factor 7-like 2), KLF4 (Kruppel-like factor 4) and EGR1 (early growth response 1) (up-regulated), and PAX6 (paired box 6) (down-regulated) were obtained in ANP_1hour. In addition, 11 TFs including ESRRG (estrogen-related receptor gamma), TGFB2 (transforming growth factor, beta 2) and HOXD13 (homeobox D13) (up-regulated), as well as down-regulated TFs including LIF (lactotransferrin/lactoferrin), ETAV7 (Ets variant 7), TNF (tumor necrosis factor) and NHLH1 (nascient helix loop helix 1) of ANP_6hours were discovered. The transcriptional regulatory network is shown in the Figure.

**Co-expressed modules:** The cluster dendrogram showed the module assignment of 479 genes in the 3 comparison groups (Supplemental Figure 3A). A total of 15 modules...
were obtained, and 3 modules including the blue, brown, and yellow modules with relatively high correlation coefficients and P values less than 0.05 were detected. Functional enrichment analysis of genes of the 3 modules are shown in Table III. Genes including CSF2 (colony stimulating factor 2) and PF4 (platelet factor 4) of the blue module were related with cytolysis, DEGs including FXYD1 (FXYD domain containing ion transport regulator 1) and TGFB2 (transforming growth factor, beta 2) of the yellow module which showed significantly low expression in ANP_0hour were mainly enriched in ion transport and the ovulation cycle. The expression level changes of genes in each module are shown as a heat map (Supplemental Figure 3B, C and D). We observed a decline in expression levels of most genes in the blue module, especially that of genes in ANP_1hour and ANP_6hours. In contrast, a slightly decreased expression of genes in the brown module was also observed. In contrast, the expression of genes in the yellow module mainly increased with time after ANP treatment, and furthermore, the assignment modules of ANP_0hour and ANP_1hour were plainly distinguishable.

Expression level of DEGs measured by real-time quantitative RT-PCR: The mRNA level of TGFB2 which was up-regulated and expressed in ANP_6hours, but not in ANP_1hour analyzed by RT-PCR. The primers of TGFB2 were F: 5'-TCCGGCCACTTCTAC-3' and R: 5'-CCGT TGTTACGGCCTTC-3', and those of GAPDH were F: 5'-TGACAACCTTTGATTGGTGAAGG-3' and R: 5'-AG GCAGGGATGATGTCTGAGAG-3'. The results showed that there was no significant difference in the TGFB2 mRNA level between the ANP-treated group and the control cells after 1h treatment, while 6h after ANP treatment, TGFB2 expression was decreased more than in the control group (Supplemental Figure 4). These results were consistent with that obtained by microarray analysis.

Discussion

ANP encoded by the NPPA gene was recently found to be involved in preventing cancer recurrence through vascular endothelia cells. To expand the adaptation of ANP for cancer therapy, however, the detailed mechanism of action of ANP should be further investigated. In this study, we explored the effects of ANP on HUVEC cells at different time points, and found that the molecular mechanism underlying the impacts of ANP on endothelial cells may change over time.

Our results showed that the number of genes differentially expressed in HUVEC cells was increased over time after ANP treatment. However, rarely overlapping DEGs were present in different comparisons. GO and pathway enrichment analysis revealed that genes of different comparisons were differentially enriched. This may indicate that ANP exerts its effects on cells through different mechanisms in a time-dependent manner. In addition, up-regulated TFs including TGFB2, as well as less-expressed TFs such as LTF and TNF, were highlighted in the transcriptional regulatory network of ANP_6hours. Moreover, none of the 3 TFs were differentially expressed in ANP_0 hours or in ANP_1hour. TGFB2 in particular was found to be related with hydrolase activity, cell morphogenesis, and cytokine-cytokine receptor interaction, while LTF and TNF were associated with leukocyte motility and activation, and immune response. A previous study has suggested that endothelial cell apoptosis can be induced by
TGFB2, which also exhibits direct immunosuppressive effects to protect cells from the immune system. In contrast, LTF is an important immune modulator to induce immune responses. In addition, TNF was demonstrated to be involved in cell apoptosis and immune response regulation and has been implicated in various diseases including autoimmune diseases. Therefore, 6 hours after ANP treatment, cell apoptosis may have occurred and immune responses were suppressed, the result of which was vascular dilation. Meanwhile, TP73 was prominent in the network of ANP_0hours, and furthermore, genes that are mainly related with ion transport indicated that the epithelial ion transport processes were altered immediately after the ANP treatment. Moreover, previous studies have suggested that treating endothelial cells for 1 hour with ANP influences gene expression and endothelial permeability. In our study, multiple genes which were highlighted in the transcriptional regulatory network were found in various BP terms as well. Of particular note is TCF7L2 which is essential for the maintenance of the epithelial stem-cell compartment in zebrafish intestine. In addition, the protein coded by EGR1 supports angiogenic responses of endothelial cells, and it is important for cell differentiation and mitogenesis. For this reason, endothelial barrier function is most likely regulated by a complex interaction of genes whose expression levels changed following the 1 hour ANP incubation.

To explore the possible gene expression pattern associated with ANP treatment, modules of co-expressed genes were identified, 3 of which were significantly clustered. As a result, functional analysis illustrated some potential genes involved in the mechanism behind the effects of ANP. For example, CSF2 which encodes a cytokine is involved in the regulation of survival, production, and differentiation of various hematopoietic precursor cells, including granulocytes, eosinophils, and macrophages, as well as in the control of their functions. Granulocyte-macrophage (GM)-CSF2, which links innate and acquired immunity, plays a pathogenic role in clinical conditions. Additionally, the CXC chemokine encoded by PF4, an inhibitor of angiogenesis, hematopoiesis, and T-cell function, was demonstrated to be involved in platelet aggregation and endothelial cell proliferation. PF4 of the blue module were found to be involved in the negative regulation of cytolysis and immune response. Since lymphocyte-mediated cytolysis of cardiac myocytes has been found in myocarditis, we speculated that increased ANP may induce the cytolysis of venous endothelial cells. Moreover, a previous study found that cytolysis of vascular endothelial cells caused endothelial injury and dysfunction, which results in increased permeability of endothelium. In addition, CSF2 was regulated by the down-regulated TF of LTF, which were found to stimulate growth factor-mediated endothelial cell proliferation and migration, and this may indicate LTF has a role in cytolysis. DEGs of the yellow module which showed significantly low expression in ANP_0hour were mainly enriched in ion transport and the ovulation cycle, and this is consistent with the results of independent analysis of DEGs in this comparison.

Additionally, to validate the accuracy of microarray data, quantitative real time RT-PCR analysis of TGFB2 transcript expression was conducted. The results showed that TGFB2 was not significantly changed in ANP_1hour, but was up-regulated in ANP_6hours. This indicated the reliability of the microarray analysis to some extent.

### Conclusions

Combining separate analysis and integrated analysis of DEGs in each comparative group, cell apoptosis may occur and immune responses would be suppressed to cause endothelial dysfunction following 6 hours of ANP incubation. In addition, ion transport was affected promptly after ANP treatment. Therefore, increased ANP may promote the cytolysis of vein endothelial cells, and then endothelial permeability would be enhanced and followed by activated immune responses. However, exactly how signaling is initiated remains unknown. Therefore, further study is needed to validate and strengthen our study results with respect to the precise mechanism underlying ANP-based treatment in cancers.
Disclosures

Conflicts of interest: The authors declare that they have no competing interests.

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Supplemental Files

Supplemental Figures 1-4

Please find supplemental files; https://doi.org/10.1536/ihj.16-522