行政院國家科學委員會補助專題研究計畫成果報告

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※　利用微矩陣研究糖尿病小鼠心臟之基因表現型態　※

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計畫類別：■個別型計畫　　□整合型計畫

計畫編號：NSC90－2314－B－002－218

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計畫主持人：王宗道

共同主持人：李源德

計畫參與人員：陳惠文，陳建尉

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□赴國外出差或研習心得報告一份

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□國際合作研究計畫國外研究報告書一份

執行單位：臺大醫院內科部心臟內科

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行政院國家科學委員會專題研究計畫成果報告

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主持人：王宗道　臺大醫院小兒科部心臟小兒科
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1. Introduction

Diabetes mellitus is one of the leading public health problems in the industrialized world, and it has a profound effect on the cardiovascular system. Most of the increased morbidity from diabetes is related to cardiovascular dysfunction. Several clinical and experimental studies, as well as our previous observation, have established that chronic diabetes mellitus can negatively alter myocardial function independent of vascular defects [1,2]. At the cellular level, the functional impairment is observed as a decreased amplitude and time course of myocyte contraction, as well as abnormal calcium cycling. Furthermore, abnormal cytoskeletal remodeling, attenuated inotropic response to β-adrenergic stimulation, impaired metabolism, and altered protein turnover are also correlated with development of heart failure [3].

Because heart failure is a multifactorial disease, expression of multiple clusters of genes is likely to be altered [4]. Large-scale sequencing studies have shown that the frequencies of several expressed sequence tags (ESTs) differ in cDNA libraries from hypertrophic versus nonfailing hearts [5]. Recent technological advances in the production of cDNA microarrays have made it possible to profile gene expression of tens of thousands of genes simultaneously. High-density arrays of cDNA inserts are produced on nylon membranes or glass slides by high-speed robotic printing [6,7]. Each printed cDNA insert on the microarray is suitable for molecular hybridization, thus allowing rapid assessment of mRNA expression of all arrayed genes in tissues of interest. Differential patterns of gene transcription by microarray analysis have also been reported in animal models of cardiomyopathy and myocardial infarction [8,9]. However, quantitative measurements of changes in expression of large numbers of genes in diabetic hearts have not been reported, despite diabetic cardiomyopathy is an important and well recognized cause of heart failure.

We herein used cDNA microarray technology to identify gene expression changes in hearts of streptozotocin-induced diabetic mice. Approximately 9000 cDNAs collected from mice cDNA libraries were printed onto microarrays and profiled for expression in the left ventricular myocardium at 2, 4, and 8 weeks after streptozotocin injection. Altered expression of the genes identified in this study may contribute to development of heart failure and provide us insight regarding the potential gene therapy for heart failure.

2. Methods

1. Animal model of diabetes: Male BALB/C mice weighing ~50 g are randomly separated into control and experimental groups. After a 7-day adaptation period, diabetes mellitus is induced by injection of streptozotocin (75 mg/kg ip) diluted in 1 ml of saline. Control animals receive a similar injection of the vehicle alone. These animals are maintained on normal chow and water ad libitum. Two days after injection, induction of the diabetic condition is confirmed by measurement of glucose concentration in blood samples. Mice rendered diabetic are studied at 1, 2, 4, and 8 weeks after streptozotocin injection. All experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996). The experimental protocol is approved by the Institutional Animal Care and Use Committee.

2. Non-isotopic labeling of heart mRNA from control and streptozotocin-induced diabetic mice: Total RNA was extracted from hearts of control and diabetic mice. The tissue was homogenized in 3 ml of solution A containing 4M guanidine thiocyanate, 0.5% sarcosyl, 25 mM sodium citrate, and 0.1 M beta-mercaptoethanol at pH 7.0, followed by phenol extraction, isopropanol precipitation, and ethanol precipitation. Quality of RNA was examined by agarose gel electrophoresis. Messenger RNA was purified using Qiagen Oligotex extraction kit. Five micrograms of mRNA was annealed with 6μM random hexamer in a total volume of 50 μl. The cDNA synthesis was performed in a 100 ml mixture contining 0.5 mM each dATP, dCTP, dGTP;40 mM dTTP, 40 mM biotin-16-dUTP (Boehringer Mannheim), 10 mM DTT; 0.5 units/ml Human Placental Ribonuclease Inhibitor (HT Biotechnology Ltd., UK), and 200 units of Superscript RT II.

3. Microarray system: The whole system included tool pin, adapter, washing slots, sampling slots, sample distribution region, program and automatically changing plate system. Those treated pins were fitted into multiple-pore adapter, which is designed specially to make the pins elastic while spotting samples on membrane. The PCR-amplified cDNA in V-bottomed 96-well microtiter plates were distributed onto a positively charged nylon membrane with spots spaced 150 ~ 500 μm apart by an arraying machine. Every time after DNA was spotted on membranes, by means of movement of translation stage, the solid pin was immersed into washing slots, and washed by 95% ethanol, and deionized water respectively with sonicating. The nylon membranes (measured 18 mm by 27 mm) carrying 9,600 PCR-amplified cDNA fragments were prepared by arraying machine built in house. The cDNA microarray membrane contains 9,600 non-redundant expressed sequence tag (EST) clones with putative gene names collected randomly from a normalized male mice cDNA library.

4. Microarray hybridization: The membrane carrying the double-stranded cDNA targets was pre-hybridized in 3 ml (option) hybridization buffer (5× SSC, 0.1 % N-lauroylsarcosine, 0.1% SDS, 1% blocking reagent made by Roche Molecular

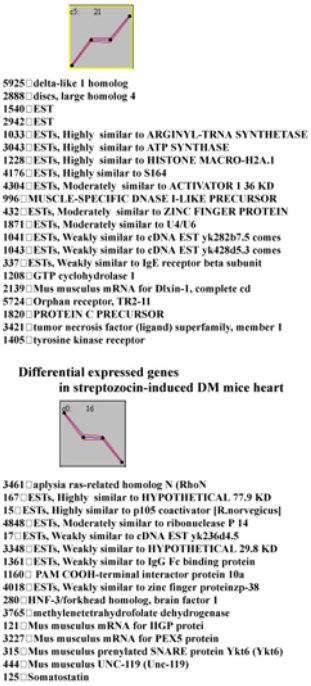
Biochemicals, and 50 μg/ml salmon sperm DNA) at 68°C for 1 hour before hybridization was carried out. cDNA probes (up to 5μg) were resuspended in 100 μl (option) hybridization buffer containing 200 μg/ml $d(A)_{10}$ and 300 ~ 400 μg/ml human COT-1 DNA (GIBCO-BRL) to prevent non-specific binding and were hybridized to the cDNA fragments on the membrane by Southern hybridization procedure.

5. Data analysis: The images captured by a scanner could be digitized by a commercial analysis software such as GenePix 3.0 (Axon instruments) or the program written in-house. Before digitization, the image produced from colorimetry detection was transformed to gray scale first, and then was digitized by analysis software. The intensity value of spots on each membrane or glass were re-scaling, log transformation, mean/median center and normalization. The pre-treated data were then clustered by hierarchical method or self-organizing maps. These softwares were free and available from Stanford university and Massachusctts Institute of Technology once got the license permission for non-profit use.

6. Cluster analysis of gene expression patterns: Genes were clustered into groups on the basis of expression patterns by SOMs (self-organizing maps). The 9,600 putative genes on each of all membranes were re-scaled first by intensity, then were filtered to remove those genes with zero or unchanged value across all time points. And then genes were performed mean center to make the mean value equal to zero, and followed by normalization to make the magnitude equal to one (sum of the squares of the values). After cluster analysis by SOM method, all of the genes of the expression patterns with ascending and descending trend responded to P53MRD were identified, and the selected genes were clustered again by hierarchical cluster method. The clustered genes were grouped into categories on the basis of our knowledge by their likely function. Some genes with multiple roles were included in more than one category.

3. Results and Discussions

We herein use cDNA microarray technology to identify gene expression changes in hearts of streptozotocin-induced diabetic mice. Approximately 9000 cDNAs collected from mice cDNA libraries were printed onto microarrays and profiled for expression in the left ventricular myocardium at 2, 4, and 8 weeks after streptozotocin injection. We identified 21 up-related genes, of which the expressions were persistently escalated in the defined time points (Figure, upper panel). On the other hand, we identified 16 down-regulated genes, of which the expressions were persistently declined in the four defined time points (Figure, lower panel). To confirm the results derived from microarray approach, differentially expressed clones were individually amplified by PCR to serve as probes for Northern blotting. Altered expressions of genes were further demonstrated by Northern hybridization. Altered expression of the genes identified in this study may contribute to development of heart failure and provide us basis for constructing strategies for gene therapy.



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