Pharmacogenomics of Cardiovascular Pharmacology: Pharmacogenomic Network of Cardiovascular Disease Models

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Received March 12, 2007; Accepted March 26, 2008

Abstract. The most important strategies in pharmacogenomics are gene expression profiling and the network analysis of human disease models. We have previously discovered novel drug target candidates in cardiovascular diseases through investigations of these pharmacogenomics. The significant induction of S100C mRNA and protein expression was detected in the rat pulmonary hypertension and myocardial infarction model. We also found increased taurine in hypoxia, a calcium-associated cytoprotective compound, to suppress the hypoxia-induced S100C gene expression and vascular remodeling. These results suggest that S100C may be one of the potential novel drug targets in hypoxic or ischemic diseases. Delayed cerebral vasospasm after aneurysmal subarachnoid hemorrhage causes cerebral ischemia and infarction. Using a DNA microarray, a prominent upregulation of heme oxygenase-1 (HO-1) and heat shock protein (HSP) 72 mRNAs were observed in the basilar artery of a murine vasospasm model. Antisense HO-1 and HSP 72 oligodeoxynucleotide inhibited HO-1 and HSP 72 induction, respectively, and significantly aggravated cerebral vasospasm. Moreover, we have also developed a unique heart failure model in zebrafish and identified several candidate genes as novel drug targets. These results suggest that pharmacogenomic network analysis has the potential to bridge the gap between in vitro and in vivo studies and could define strategies for identifying novel drug targets in various cardiovascular diseases.

Keywords: pharmacogenomics, network analysis, transcriptome, DNA microarray, therapeutic gene

1. Drug target screening and validation by pharmacogenomics

Pharmacogenomics is defined as the identification of the genes or loci that are involved in determining the responsiveness and to distinguish the responders and non-responders to a given drug (1). Genome sequencing, transcriptome, proteome, metabolome, and network analysis (2) are of particular significance in pharmacogenomics. Sequencing is used to locate polymorphisms, and monitoring of gene expression can provide clues about the pharmacological genomic response to disease and treatment. The transcriptome analysis can be done by methods of random cDNA sequencing, mRNA display (differential display, fluorescent differential display, RNA arbitrarily primed PCR, molecular indexing, gene expression fingerprinting, etc.) and differential hybridization (cDNA high density filter, cDNA microarray, oligomicrochip, etc.). We used a transcriptome analysis to screen therapeutic target genes by studying the change of gene expression in animal models of pulmonary hypertension, cerebral vasospasm, and heart failure and found novel drug target candidates through this pharmacogenomic strategy (3 – 7). These pharmacogenomics classify genomes into 4 kinds of genes such as a disease-associated gene cluster, drug-associated gene cluster, therapeutic genes, and other genes (Fig. 1).
2. Target validation of pulmonary hypertension model

Gene expression profiling through pharmacogenomic transcriptome and network analysis can identify novel therapeutic genes in pulmonary hypertension. Furthermore, the simultaneous identification and quantification of biogenetically synthesized metabolites (metabolome) highlights the molecular mechanisms, providing a link between low-molecular-weight metabolites and the expression of genes or proteins.

Exposure to chronic hypoxia results in sustained pulmonary hypertension secondary to muscularization and medial hypertrophy of the pulmonary vasculature. However, the mechanism by which this occurs is not clear. Therefore, the goal of the pharmacogenomics study was to utilize a transcriptome and metabolome analysis to identify a therapeutic low-molecular-weight ligand involved in hypoxia-induced vascular remodeling.

Exposure to hypoxic conditions resulted in the up-regulation of S100C mRNA and protein associated with an increase of taurine (2-aminoethanesulfonic acid) content in rat lung. Hypoxia resulted in transcriptional activation of the S100C promoter (4, 5) through hypoxia-inducible factor-1 (HIF-1). Taurine suppressed HIF-1-mediated increases in S100C transcription. Moreover, oral taurine administration attenuated vascular remodeling in hypoxic rat lung, whereas depletion of endogenous taurine by administration of beta-alanine resulted in increased vascular remodeling. The inhibition of HIF transcription by taurine may be of therapeutic benefit in preventing hypoxia-induced vascular remodeling. These integrated strategies provided an excellent strategy for the screening and validation of therapeutic targets (3).

3. Rat model of delayed cerebral vasospasm

Delayed cerebral vasospasm after an aneurysmal subarachnoid hemorrhage (SAH) causes cerebral ischemia and infarction. We performed a transcriptome and network analysis to identify therapeutic target genes in the rat model of SAH.

Despite considerable advances in diagnostic, surgical, and anesthetic research and perioperative management, the outcome for patients with SAH remains poor. Cerebral vasospasm is the delayed narrowing of large capacitance arteries at the base of the brain and is a major cause of morbidity and mortality after SAH. In about one half of cases, a delayed neurological ischemic deficit occurs, and 15% – 20% of such patients suffer stroke or die from vasospasm despite maximal therapy. Accumulating evidence suggests that hemoglobin (Hb)-induced oxidative stress plays a central role in the pathogenesis of vasospasm after SAH. However, the mechanism by which delayed cerebral vasospasm resolves spontaneously has not been sufficiently investigated. If the intrinsic mechanism of spasmolysis was clarified, then it might lead to a novel therapeutic strategy. We employed an improved differential display technique, fluorescent differential display (FDD), and DNA microarray to identify differentially expressed genes and evaluate the functional significance of such genes in the basilar artery of a rat vasospasm model (6, 7).
4. Target validation for cerebral vasospasm

The rats were allocated into 1 of 2 groups: a hemorrhage group and a saline-injected group. The subarachnoid hemorrhage rats were given injections of autologous blood into the cisterna magna. The saline group was given saline injection by the same protocol. The subarachnoid hemorrhage rats showed biphasic vasospasms that occurred at 10 min (early vasospasm) and on Day 2 and Day 7 (delayed vasospasm) after blood injections. The delayed vasospasm in the hemorrhage rats resembled human vasospasm. No significant vasospasm was observed in the saline group (6).

From the FDD fingerprints, we identified changes in intensity in approximately 3% (280 bands) of the 9642 bands specific for delayed vasospasm in the hemorrhage rats. One upregulated cDNA fragment which coincided with the development of vasospasm proved to be the rat HO-1 gene. Therefore, we focused on this gene to clarify the target validation for vasospasm.

The HO-1 mRNA levels in the basilar artery and brain obtained at identical time points were determined by the quantitative RT-PCR analysis method. Real time RT-PCR analysis demonstrated that HO-1 mRNA was exclusively induced in the basilar artery and modestly induced in the whole brain in the SAH group, but not in the saline group. Interestingly, the expression levels of HO-1 mRNA in the basilar artery were the most prominent among the tissues evaluated. In spite of the identical distribution of SAH, the levels of HO-1 mRNA in the basilar artery with delayed vasospasm were about 7 times higher than in the ventral brain stem on Day 7 in the hemorrhage group. A significant linear correlation was observed between the degree of delayed vasospasm and the HO-1 mRNA levels in the basilar artery, ventral brain stem, and cerebellum. HO-1 mRNA was not induced in the basilar artery with early vasospasm or in the brain 10 min after the blood injection on Day 0.

To determine the functional significance of HO-1 gene induction in the basilar artery and brain, we examined the effects of selective HO-1 inhibition using antisense HO-1 oligodeoxynucleotide (ODN) on angiographic vasospasm. In the basilar artery, a successful blockage of HO-1 mRNA upregulation was observed after 2 injections of antisense HO-1 ODN. The HO-1 antisense ODN also prevented the upregulation of HO-1 protein in the basilar artery. The levels of HO-2 and β-actin mRNA were not affected by antisense HO-1 ODN, sense HO-1 ODN, or scrambled ODN treatment. The sense HO-1 ODN or scrambled ODN treatment did not affect the HO-1 gene expression levels and delayed vasospasm in the basilar artery. On the other hand, the antisense HO-1 ODN significantly inhibited HO-1 induction in the basilar artery until Day 4 after SAH. Moreover, the antisense HO-1 ODN significantly aggrivated the vasospasm. The peak time of vasospasm was delayed from Day 2 to Day 4. Vasospasm was present up until Day 7, when it had already resolved in the control rats. The magnitude of HO-1 induction in the whole brain tissue was much smaller than in the basilar artery. These results suggest that the HO-1 levels in the whole brain tissue may reflect the HO-1 induction in the neuronal tissue that was not affected by antisense HO-1 ODN. In the neuronal tissue, HO-1 induction has been observed in glial cells after SAH. Probably, the inhibitory effects of antisense HO-1 ODN treatment on HO-1 induction in the basilar artery might be masked by the greater amounts of HO-1 induction in glial cells. Therefore, the intrathecal injections of antisense HO-1 ODN affected the HO-1 levels in the basilar artery, but not in the neuronal tissue (6).

5. HO-1 gene induction as a novel drug target

Recent studies support the hypothesis that HO-1 induction plays an important role in cellular protection against both heme- and non-heme-mediated oxidative injury. This study demonstrated the upregulation of HO-1 mRNA in the cerebral arteries and, more importantly, the augmentation and prolongation of delayed vasospasm by antisense HO-1 ODN treatment after SAH (6). Moreover, we found the protective effects of HO-1 gene induction by an endogenous or clinical compound, nicaraven (AVS), in cerebral vasospasm (8).

HO is the rate-limiting enzyme in the heme degradative pathway. All isoforms of HO, HO-1 (an inducible isozyme), HO-2 (a non-inducible isozyme), and HO-3 (a recently identified isozyme), metabolize heme in Hb and generate carbon monoxide (CO), free iron (ferric iron), and biliverdin (subsequently reduced to bilirubin). The induction of HO can have dual beneficial effects against the toxicities of Hb: namely, it removes Hb itself and synthesizes an antioxidant bilirubin.

In the vascular cells, the expressions of HO-1 have been observed in the endothelial and smooth muscle cells in some pathophysiological conditions. CO, a by-product of HO, is a gas molecule that shares some of the properties of nitric oxide, inasmuch as CO binds to the heme moiety of cytosolic guanylyl cyclase to produce cGMP. In the vascular smooth muscle cells, HO-1 is induced by hypoxia and its by-product, CO, promotes the accumulation of cGMP. Increased cGMP causes smooth-muscle relaxation. Furthermore, smooth muscle cell-derived CO inhibits the production of the endothelium-derived vasoactive agents, endothelin-1 and platelet-derived growth factor-B.
We found a remarkable upregulation of HO-1 mRNA in the basilar artery which might be closely related to the occurrence of delayed vasospasm after SAH. We clearly demonstrated that the intrathecal administration of antisense HO-1 ODN aggravates vasospasm, thus suggesting that HO-1 gene induction has spasmolytic effects. Furthermore, we found the protective effects of HO-1 gene induction by an endogenous or clinical compound, nicaraven (AVS), in cerebral vasospasm (8). The therapeutic gene induction of HO-1 could be a novel strategy for the prevention and treatment of Hb-induced pathologic conditions including delayed cerebral vasospasm (Fig. 2).

6. Heat shock protein 72 induction as novel drug target

Using a DNA microarray system, we identified the gene encoding for Heat Shock Protein 72 (HSP72) to be highly up-regulated by delayed cerebral vasospasm (7). We therefore elucidated the role of the HSP72 gene in cerebral vasospasm using a rat experimental SAH model. Using angiography, cerebral vasospasm was detected from Day 1 with maximal narrowing detected on Day 2. The intracisternal injection of antisense HSP72 ODN led to specific inhibition of HSP72 gene expression and significantly aggravating cerebral vasospasm on Days 2 and 3 of the angiographic studies. The oral administration of geranylgeranylacetone (GGA), an anti-ulcer drug, enhanced HSP72 induction and reduced cerebral vasospasm (7). These results suggest that HSP72 plays a novel role in antagonizing delayed cerebral vasospasm after SAH, while GGA also induces a protective effect against delayed cerebral vasospasm, at least partly via the induction of HSP72 (Fig. 3).

Real-time RT-PCR showed a greater than 8.0-fold up-regulation of HSP72 mRNA on Day 2 after SAH in comparison to the no-treatment group and a 1.9-fold up-regulation of HSP72 mRNA on Day 2 after SAH in comparison to the results of the saline injection group. Constitutive HSP73 mRNA after SAH was unchanged in comparison to the levels measured after saline injection. Real-time RT-PCR also showed a notable induction (1.8-fold) of HSP72 mRNA expression on Day 2 after SAH. HSP72 protein induction was determined by Western blotting using an antibody that specifically recognizes inducible HSP72. A significant induction (2.7-fold) of HSP72 protein was detected on Day 2 after SAH in comparison to that seen on Day 2 after saline injection.

An angiographic examination of cerebral vasospasm after SAH in the basilar artery was performed. In the SAH group of rats, cerebral vasospasm was detected from Day 1, with the maximal vasospasm occurring on Day 2. The average arterial diameter decreased to 86% of that seen in the baseline angiograms. The vasospasm then slowly abated and was gone by Day 7. In the saline group, no significant difference in the basilar artery diameter was observed for either group between the Day 2 measurement and the baseline angiogram.

To determine the functional significance of the induction of the HSP72 gene in the basilar artery, we
examined the effect on vasospasm of selective HSP72 inhibition using antisense HSP72 ODN. RT-PCR showed that a successful inhibition of HSP72 mRNA up-regulation was observed on Day 2 after intracisternal injections of antisense HSP72 ODN. Real-time RT-PCR showed notable inhibition (0.74-fold) of HSP72 mRNA expression on Day 2 after intracisternal injections of antisense HSP72 ODN. The antisense HSP72 ODN was also found to significantly reduce up-regulation of the HSP72 protein. A densitometric analysis demonstrated that antisense HSP72 ODN decreased the level of HSP72 protein to 54.1% of the control value obtained with sense HSP72 ODN.

Significant differences in the arterial diameters measured by angiography were apparent on Days 2 and 3 after intracisternal injection of antisense HSP72 ODN or sense HSP72 ODN. In this way, intracisternal injections of antisense HSP72 ODN significantly aggravated the extent of vasospasm measured angiographically on Days 1 to 5 following SAH. No differences in the extent of vasospasm were observed when the animals were treated with sense HSP72 ODN injection in comparison to SAH. The vasospasm gradually subsided until Day 7 in the group injected with antisense HSP72 ODN.

In addition to the above experiments, we found that GGA induced HSP72 expression in the brain and basilar artery. The dose-dependent effects of GGA on the expression of HSP72 in the brain and basilar artery were assessed 24 h after a single oral administration of GGA. The HSP72 expression in both the brain and basilar artery was observed for doses in excess of 400 mg/kg and increased in a dose-dependent manner up to 600 mg/kg. The time-dependent effects of GGA on the expression of HSP72 in the brain and basilar artery were also evaluated after a single dose administration of GGA (400 mg/kg). The HSP72 expression in the brain and basilar artery was observed from 4 h and then slowly decreased and had disappeared by 48 h.

The levels of HSP72 in the basilar artery were examined by RT-PCR and a Western blot analysis after saline injection or SAH with oral administration of GGA or its vehicle only. The overexpression of HSP72 mRNA by the end of Day 2 after SAH or saline injection following 4 oral GGA boluses, administered every 12 h, was dose-dependently increased in comparison to that after SAH or saline injection with just 2 orally administered GGA boluses on Day 1. Furthermore, on Day 2, the overexpression of HSP72 mRNA after SAH with oral GGA treatment was significantly enhanced in comparison to that after SAH with vehicle treatment and after saline injection with GGA treatment.

The comparisons of the arterial diameters measured by angiography on Days 1, 2, and 3 after SAH with the oral administration of GGA showed significant improvement in comparison to when GGA was not administered.
Based on the above findings, HSP72 induction by GGA is therefore proposed to be an intrinsic protective mechanism that is considered to be a potential therapeutic target for the treatment of cerebral vasospasm (Fig. 3).

7. Zebrafish as a novel human disease model

The zebrafish is becoming an increasingly popular model organism to study pharmacogenomics and network analysis. Novel assays have been and continue to be developed to identify novel drug targets using the zebrafish. Methods to validate potential drug targets include morpholino antisense knockdown technology and target-selected mutagenesis approaches.

The zebrafish is becoming increasingly popular among scientists from diverse fields such as pharmacogenomics and toxicogenomics. Researchers from academic institutions and pharmaceutical companies regard the zebrafish as a new powerful tool in drug discovery that will accelerate screening processes (9).

Zebrafish possess many features that make them a highly useful model system. The adults are relatively small (only 3 – 4 cm-long), inexpensive, easily housed, and maintained in large quantities. Zebrafish can be easily mated under laboratory-controlled conditions, with each pair typically producing several hundred eggs per mating. The embryos are transparent, allowing easy visualization of fundamental developmental processes. They develop rapidly: a one-day-old embryo already has formed all the major tissues and many organ precursors, such as a beating heart, circulating blood, nervous system, eyes, and ears, all of which can be readily observed under a simple dissecting microscope. By 5 days post-fertilization (5 dpf), the zebrafish larva has formed the majority of internal organs including the liver, pancreas, and a complex vascular network. These features, in addition to the relatively short generation time of approximately three months, make genetic screens feasible. Given these attributes, experiments in zebrafish are significantly easier, faster, and cheaper than in mice, which is currently one of the main model systems for drug target discovery and validation.

Although assay development to model human diseases in zebrafish is still in its infancy, a variety of diseases have so far been successfully modeled in zebrafish. For example, we developed a cardiac failure model of zebrafish. The development of in vivo assays that are related to human heart failure could lead to the discovery of novel potential drug targets.

8. Development of a human disease model of zebrafish

Reverse genetics approaches can be used to validate novel drug targets in human disease models (Fig. 4). There is a main method under development in this area: the use of antisense molecules to knockdown the function of a gene of interest. The fastest way to analyze the function of a gene of interest is by using antisense methods. From several different antisense molecules that have been tested, morpholino phosphorodiamidate oligonucleotides (morpholinos [MOS]); and more recently, peptide nucleic acids (PNAs) have been demonstrated to effectively knockdown a specific gene function in zebrafish. MOs have received particularly wide usage owing to their high efficacy, specificity, and commercial availability. Translation-blocking MOs are designed to target the 5' untranslated region (UTR) of mRNA or the first 20 – 25 nucleotides of the open reading frame (ORF) of an mRNA of interest, thus preventing the initiation of translation. MOs can also be designed against splice-accept or or splice-donor sites of pre-mRNA. The splice-blocking MOs interfere with the function of the spliceosome leading to exon-skipping or the usage of cryptic splice sites and resulting in aberrantly spliced mRNA.

The ease of delivering MOs and small molecular weight chemicals and observing the resulting phenotypes in zebrafish has led to the idea of MO- and small molecule-based screens.

An alternative to mutagenesis is the use of small-molecule developmental screens. Chemical compounds of relatively small molecular weight (small molecules) can bind to specific proteins, thus modulating their functions and resulting in changes in an organism’s phenotype in a nonheritable manner. Subsequently, the
proteins that interact with the compound can be isolated using affinity chromatography methods. The structure of the small molecule can be directly used in the drug design and testing. Small molecules have been used in several pilot screens in zebrafish to assess their pharmacological effects on disease models. Small molecules have been discovered that specifically affect the brain, notochord, heart, ear development, and pigment cell formation in zebrafish embryos. We found that our heart failure model of zebrafish can be treated by the clinical therapeutics for human heart failure. These studies illustrate that small-molecule screening can therefore be a powerful method to identify novel drug targets.

At this point, the zebrafish genome sequencing project at the Sanger Centre is under way and is expected to be completed in 2008. More than 1,370,000 expressed sequence tags (ESTs) have been sequenced. The analysis of gene function is greatly aided by a microarray analysis. Two different zebrafish oligonucleotide-based microarrays are currently available, one designed by Agilent Technologies that we used and the other by Affymetrix, and each contain over 21,000 unique sequences.

The zebrafish has come a long way from an aquarium fish to becoming a favorite model of choice in pharmacogenomics. Owing to recent advances in zebrafish-based assay development, novel mutagenesis tools and progress in genome analysis, the zebrafish is fast gaining popularity for modeling human diseases and drug development. The potential for understanding the underlying mechanisms of many human diseases and development of novel drugs to fight them will soon be a reality with the help of the zebrafish. The zebrafish model system is therefore considered to offer great advantages in the integration of reverse and forward pharmacogenomics (Fig. 4).

9. Conclusion

Our results suggest that pharmacogenomics, especially transcriptome and the network analysis of a human disease model of the rat and zebrafish, has the potential to bridge the gap between in vitro and in vivo studies, thus effectively helping to define strategies to identify novel drug targets for the treatment of various cardiovascular diseases.

Acknowledgments

This work was supported in part by Grants from the New Energy and Industrial Technology Development Organization; Grants-in-Aid for Scientific Research and for International Scientific Research from the Ministry of Education, Science, Sports, and Culture; the Program for the Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research; and the Grant for Pediatric Diseases from the Ministry of Health and Welfare, Japan. It was also supported in part by Grants-in-Aid for the Research Projects from the Mie Medical Research Foundation. We also thank Ms. K. Nishiguchi, Ms. C. Suzuki, Ms. Y. Yoshikawa, Ms. T. Murata, and Ms. A. Kamakura for their experimental assistance and Ms. R. Ikeyama, Ms. Y. Yoshida, and Ms. K. Ito. for secretarial assistance.

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