Gene profile for differentiation of vascular adventitial myofibroblasts

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Abstract: Our previous study demonstrated that TGF-β1 could induce the differentiation of vascular adventitial fibroblasts (AFs) to myofibroblasts (MFs). The aim of this study was to identify the genes which might be responsible for the cell phenotypic change using genechips. Cultured rat AFs were treated with TGF-β1 (10 ng/ml) for 0 min, 5 min, 15 min, 2 h, 12 h and 24 h, respectively. Then the cells were gathered to prepare total RNA. We examined TGF-β1-induced gene expression profiling using Affymetrix oligonucleotide microarrays and analyzed data by GCOS1.2 software. Moreover, expressional similarity was measured by hierarchical clustering. Some of genechip results were confirmed by real-time quantitative RT-PCR. Microarray analysis identified 2 121 genes with a 2-fold change or above after TGF-β1 stimulation. 1 318 genes showed a greater than 2-fold increase and 761 genes were reduced 2 folds or more at mRNA levels, whereas a small portion of the total regulated genes (42 genes) displayed dynamically up- and down-regulated pattern. Genes were further segregated for early (peak at 5 min, 15 min and/or 2 h), late (peak at 12 h and/or 24 h), and sustained (2-fold change or above at five time points) temporal response groups according to the time of their peak expression level. Among 1 318 up-regulated genes, 333 genes (25.3%) responded rapidly to TGF-β1 and 159 genes (12.1%) responded in a sustained manner. Most genes (826, 62.6%) were regulated at 12 h or later. For the 761 down-regulated genes, numbers of early and late responsive genes were 335 (44%) and 267 (36.1%), respectively. There were also 159 genes, 19.9% of total down-regulated genes, decreased at five time points treated by TGF-β1. The results suggested that the gene expressions of secreted phosphoprotein 1 (APP1) and Rho-associated coiled-coil forming kinase 2 (ROCK2) had the same trends as α-smooth muscle-actin, a marker of MF differentiation. In addition, the gene expression of potassium voltage-gated channel, Shal-related family and member 2 (KCND2) was up-regulated. Furthermore, it was found that endothelin 1 (EDN1), some complement components, NADPH oxidase 4 (NOX4) and NAD(P)H dehydrogenase, quinone 1 (NQO1) might be involved in MF differentiation. Using microarray technique, we confirmed some genes that have been identified by other techniques were implicated in MF differentiation and observed new genes involved in this process. Our results suggest that gene expression profiling study is helpful in identifying genes and pathways potentially involved in cell differentiation.

Key words: oligonucleotide arrays; adventitia; fibroblast; differentiation

血管外膜肌成纤维细胞分化基因表达谱

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摘 要：我们以往的研究表明, TGF-β1 可以诱导血管外膜成纤维细胞(adventitial fibroblasts, AFs)向肌成纤维细胞(myo-fibroblasts, MFs)分化。为寻找可能涉及 MF 分化的基因, 本实验采用寡核苷酸芯片技术动态检测细胞表型转化过程中基
The change of vascular structure and function caused by vascular remodeling is common physiopathological basis for the pathogenesis and development of a wide spectrum of cardiovascular disorders such as atherosclerosis and hypertension. The researches on vascular remodeling mainly focused on vascular endothelial cells and media smooth muscle cells in the past years. Nevertheless in 1991, Zhu et al.[1] first found adventitial fibroblasts (AFs) cultured in vitro from thoracic aortae of spontaneously hypertensive rats (SHR) proliferated faster than those of Wistar Kyoto (WKY) rats, and its response to many growth factors is stronger than that of the latter too. Findings in porcus model of balloon overstretch coronary artery also demonstrated that AFs proliferated earlier than vascular smooth muscle cells (VSMCs) and transformed to myofibroblasts (MFs) characterized as expressing α-smooth muscle-actin (α-SM-actin), a protein marker of MFs[2]. MFs with characters of both fibroblast cells and smooth muscle cells participated in vascular remodeling by migrating to the intima and proliferating to form neointima. What’s more, Li et al.[3] demonstrated more direct evidence that AFs participated in vascular remodeling by inducing AFs expressing LacZ stably to rat carotid sinus immediately after balloon injury and had detected LacZ gene expression in media and neointima 5~14 d after vascular injury. The phenotypic transformation from AFs to MFs stably is initial and triggering step of vascular remodeling, but the mechanism responsible for it is not clear yet. Clarifying the molecular mechanism of this process will be helpful in clarifying the advanced role of adventitial remodeling in vascular wound healing and vascular remodeling, and finding novel therapy targets for inhibiting vascular remodeling. We have successfully induced MF differentiation with transforming growth factor β1 (TGF-β1) in vitro[4]. In this study we dynamically detected the change of gene expression profiles during MF differentiation under TGF-β1 induction using oligonucleotide microarray.

1 MATERIALS AND METHODS

1.1 Cell culture
Vascular AFs were isolated from thoracic aortae of 7-week old Sprague-Dawley (SD) rats and cultured using attachment method[1]. In this study, cells were used from passage 3 to 5 after phenotypic identification[5].

1.2 TGF-β1 induction
Prior to treatment, cells were cultured in serum-free DMEM (Gibco Life Technologies) for 48 h. For temporal analysis of gene expression, cells were incubated with 10 ng/ml TGF-β1 (Valbiotech) for 0 min, 5 min, 15 min, 2 h, 12 h, and 24 h for RNA preparation.

1.3 RNA extraction
Total RNA was extracted with TRizol reagent (Gibco Life Technologies) following the manufacturer’s manual. RNA for use was quantitated by spectrophotometric analysis for 1.8~2.0 and quality was confirmed by agarose electrophoresis.

1.4 Array hybridization
Oligonucleotide chips (Rat Expression Array REA230) are commercial products from Affymetrix and technical service was provided by Genentech. The protocol was described briefly as following: synthesized double-stranded cDNA with 10 μg total RNA extracted from cells gathered at 6 time points mentioned above respectively, then purified and prepared biotin-labeled cRNA. Cleaved cRNA to fragments of 300 bp length. Six cRNAs were pre-hybridized with a test array Test 3 (Affymetrix) respectively and performed next step if all of the three following conditions are fulfilled: (1) Cy3/Cy5 of housekeeping genes β-actin and GAPDH are less than 3:1; (2) Background is less than 700; (3) Noise value is less than 10. Six cRNAs were hy-
bridized with 6 Rat Expression Array REA230 (Affymetrix) respectively. Then removed redundant hybridization solution and stained.

1.5 Data analysis
Data were collected by Scanner 3000 (Affymetrix) and analyzed using GCOS1.2 software (Affymetrix). The fluorescence signal intensities were normalized by calibrating average value of genes lived in Rat 230Anorm.msk to 2000. Compared to control array, only genes with a 2-fold change or above at least at one time point were identified as TGF-β1-responsive genes. Fold-change (increase or decrease) was calculated and transformed to log2. A hierarchical clustering algorithm was applied to log2 ratio of TGF-β1-responsive genes on the basis of expression pattern similarity over the examined time points. Expression level of each gene was represented by pseudocolor in matrix format, with red representing increasing mRNA levels and green denoting decreasing mRNA levels, and color intensity representing the magnitude of the expression ratio (Fig.1A)[6]. Gene Ontology Mining Tool (Affymetrix) was used to analyze gene function.

1.6 Fluorescent real-time polymerase chain reaction (RT-PCR)
The genes complement 3 (C3), endothelin 1 (EDN1), NADPH oxidase 4 (NOX4), NAD(P)H dehydrogenase, quinone 1 (NQO1), tropomyosin 1 (TPM1), potassium voltage gate channel, Shal-related family and member 1 (KCND1) and 3 (KCND3), identified to be regulated from the array, were analyzed using high-throughput fluorescent quantitative PCR equipment 7900HT (ABI) and SYBR premix Ex Taq Kit (TaKaRa). β-actin was used as internal primer. Primer sequences were as following: C3: forward 5’-CTACACCTCCTCCTGTTATGA-3’, downward 5’-CTGTTGAGTTTGTCTTTCT-3’; NOX1: forward 5’-GTGAGAGATGTTCTTCTAG-3’, downward 5’-AAAGTAGAGTGGTGAACCTC-3’; NOX4: forward 5’-TCTCAGAATATGTTCCATAGC-3’, downward 5’-ATACACTGGAACATGTGAA-3’; EDN1: forward 5’-AACGCCCTTTGATCTTAAAG-3’, downward 5’-GGTTGTGTATCAACTCTGTG-3’; TPM1: forward 5’-GATGAGAGTGAGAGGCAATGA-3’, downward 5’-CACGTGTTGACAGAGACTTG-3’; KCND1: forward 5’-AGCCTTTTTCTGTATGGACA-3’, downward 5’-TAGAACATGACATGGGCCA-3’; KCND3: forward 5’-TACCTACGCTCCTCAGCAGACC-3’, downward 5’-GACTCTTCCGCGCCACCAC-3’.

RT-PCR was performed in a final volume of 20 μl. Total RNA (2 μg) was reversely transcribed to cDNA and 1 μl RT product was used to amplify gene fragments[7]. Total RNA was isolated from three independent cultures and three reproducible tubes were set for each sample. Fold-changes (treated cells at 5 time points vs untreated cells respectively) were calculated and transformed to log2, as following. Ratio=2−ΔΔCt, Log2 ratio=−ΔΔCt. Compared them to corresponding microarray results.

2 RESULTS

2.1 Gene expression analysis
Affymetrix rat oligonucleotide microarray REA230 consists of 15866 probe sets. Each probe set represents one gene. Using criterion mentioned above (see materials and methods), we first identified 2121 genes displaying changes at mRNA level in response to TGF-β1, which was 13.37% of total genes in microarray. 1318 genes showed a greater than 2-fold increase and 761 genes were reduced 2 folds or more at expression level, whereas a small portion of the total regulated genes (42 genes) displayed dynamically up- and down-regulated pattern (Fig.1A).

Genes were further segregated into early (peak at 5 min, 15 min and/or 2 h), late (peak at 12 h and/or 24 h), and sustained (2-fold change or above at five time points) temporal response groups according to the time of their peak expression level. Among 1318 up-regulated genes, 333 genes (25.3%) responded rapidly to TGF-β1 and 159 genes (12.1%) responded in a sustained manner. Most genes (826, 62.6%) were regulated at 12 h or later. For the 761 down-regulated genes, numbers of early and late responsive genes were 335 (44%) and 267 (36.1%), respectively. There were also 159 genes, 19.9% of total down-regulated genes, decreased at five time points treated by TGF-β1. The expression patterns of genes in six groups mentioned above are shown in Fig.1B.

2.2 Gene function analysis
Among 2121 TGF-β1-responsive genes, biological characteristics of 1231 genes were definite and the functions of these genes were analyzed (Fig.2)

The genes responding strongly to TGF-β1 included genes which have been found to be related to MF differentiation in our previous studies, such as ROCK2 (data not shown), SPP[8,9] and KCND2 (Kv4.2)[10,11]. Changes of SPP1 and ROCK2 were the same as that of α-SM-actin. KCND2, encoding A type potassium electric current, expressed at 24 h after TGF-β1 treatment while it expressed negatively before treatment just as our previous observation. Furthermore, KCND1 and KCND3 also appeared in cells treated by TGF-β1 for 5 min, 15 min, and 2 h in early responsive manner, then expression of KCND1 had never been detected and KCND3 was detectable again at 24 h after TGF-β1
Moreover, genes encoding complement C3, C1s, C1r, H factor sustainedly increased response to TGF-β1, among which expression of C3 changed most remarkably (Fig. 3). On the other hand, expression of decay accelerating factor 1 which acts as enzyme inhibitor, was down-regulated strikingly (Fig.3), thus it seemed that the complement system presented an activated state during MF differentiation. Expression of genes encoding EDN1 and NOX4 were up-regulated persistently after TGF-β1 stimulation, and expression of EDN1 was up-regulated almost 10 folds at 12 h and 24 h after TGF-β1 stimulation. The other gene NQO1, a member of oxidative stress pathway, was down-regulated persistently by TGF-β1.

2.3 Results of real-time fluorescent quantitative polymerase chain reaction
In order to verify the data generated from the microarray, we have analyzed a number of genes identified to be regulated persistently after TGF-β1 stimulation, and expression of EDN1 was up-regulated almost 10 folds at 12 h and 24 h after TGF-β1 stimulation. The other gene NQO1, a member of oxidative stress pathway, was down-regulated persistently by TGF-β1.
lated by TGF-β1 using real-time quantitative RT-PCR. Except for NOX4, expression profiles of several other genes tested by RT-PCR are consistent with those tested by microarray.
3 DISCUSSION

It was found that accompanied by transforming their phenotype to MFs, activated fibroblasts proliferated, migrated, and then closed wound by contracting and synthesizing extracellular matrix when Gabbiani et al. investigated wound healing of rats and human beings in 1971. The same repair phenomenon also occurs during vascular injury. The active cells responsive to vascular injury, which possess features of proliferating, migrating, adhering, synthesizing extracellular matrix and expressing α-SM-actin, mostly distribute in vascular adventitia. Many growth factors and vasoactive substances such as angiotensin II (AngII), aldosterone and others can induce AFs to active MFs. TGF-β1 is the most important growth factor responsible for MF formation. It can enhance α-SM-actin expression in AFs and make them exhibit MF characters in serum-free media, which becomes an excellent model for discovering the mechanism of MF differentiation in vitro.

We dynamically detected changes of gene expression profiles during MF differentiation induced by TGF-β1. Some pathways minority genes involved in are already clear. Expression profiles of 16 genes in myometrial contraction and relaxation pathways changed after TGF-β1 stimulation. Alcitolin α decrease sustainedly plays a role of negative regulation in relaxation pathway, and later up-regulation of insulin-like growth factor binding protein 2 and 3 in relaxation pathway maybe promote cell proliferation during MF differentiation. Adenylate cyclase 2 and 5 increased 2 folds at 2 h post TGF-β1 stimulation and dropped to the normal level later, which didn’t seem to contribute much to the pathway. In muscle contraction pathway, Jun and calponin 1, which regulate smooth muscle contraction, changed in similar trends to that of the marker gene, α-SM-actin. IL-6 in this pathway increased obviously at all times, which also prompts activation of the contraction pathways. Changes of genes in muscle contraction and relaxation pathway mainly showed negative control of relaxation pathway and activation of contraction pathway. Besides, gene encoding PKC protein which locates in the central position of contraction pathway was not in the probe set list of chips we used. However, it was demonstrated that during MF differentiation induced by TGF-β1, both protein level and enzyme activity of PKC-α increased obviously[5]. The activation of contraction pathway mainly contributed to prompting MFs to acquire functional characteristic of VSMCs. Moreover, 14 genes involved in cell cycle pathways expressed differently in the cells incubated with TGF-β1, and changes of them were similar to that of α-SM-actin except that Gadd45a with transient change differed from the marker gene at 15 min after TGF-β1 treatment. Changes of genes in cell cycle pathway responded to TGF-β1 indicate MFs presented gene expression pattern which avail cells of entering DNA synthesis phase. Besides genes required by pathways mentioned, several genes in TGF-β pathway and integrin-induced cell adhesion pathway, including SPP1 and ROCK2, were also regulated by TGF-β1. Our researches showed that SPP1 not only presented the same expression pattern as α-SM-actin did in MFs, but also promoted proliferation and migration of MFs in a time- and concentration-dependent ways[8,9]. In vascular injury models, its antisense ODN inhibited formation of vascular neo-intima and thickening of vascular wall (data not shown), which suggested that SPP1 might be a potential therapy target for inhibiting vascular remodeling. The precise mechanisms of these two pathways in MF differentiation need to be elucidated further.

It is worth mentioning that the research results of gene chips not only confirmed our present knowledge of MF differentiation, but also gave new clues. For example, activation of complement system during MF differentiation, especially the significant up-regulation of C3 was discovered for the first time during MF differentiation.

Complement C3 is an immemorial immunological molecule, which plays a central role in immune defense, regulation and pathology. However, it is revealed recently that C3 protein is not only recruited upon infection and inflammation as an immune defense mechanism, but also contributes to some non-inflammatory processes such as bone development[12,13] and stem cell differentiation[14]. Strikingly, C3 is over-expressed in regenerating limb and lens during urodele axolotl’s limb regeneration[15,16]. Furthermore, C3 is required in hepatic regeneration of mammals, and exerts biologic actions attributed to cell growth[17,18]. In vessels, several papers reported that vascular endothelial cells could synthesize and secret immune molecules such as C3, etc[19] and participate in inflammatory responses. It was also discovered that C3 was produced only by VSMCs from SHR and C3 increased in VSMCs incubated with AngII as well. Meanwhile, exogenous C3 transformed VSMCs to synthetic-type phenotype and exaggerated growth of VSMCs from SHR[20]. Furthermore, it was reported by Buono et al.[21] that less VSMCs and collagen were detected in aortae from low-density lipoprotein receptor (ldlr)−/−C3-deficient mice (ldlr−/−C3−/−) versus ldlr−/−C3+/− littermate control mice. Not only is it another proof that C3 could promote proliferation of VSMCs, but also indicates that C3 might be related to collagen synthesis. Whether vascular AFs could secret C3 has not been documented yet.

Did up-regulation of complement elements, especially C3, after TGF-β1 stimulation in AFs suggest that MF differentiation requires an immunological mechanism? Or has C3 complement played novel non-inflammatory functions which have not yet been known in vascular wound healing? These questions provide new clues for advanced research.
Interestingly, expression profile changes of EDN1 and NOX4 also inspired different points on MF differentiation mechanism. EDN1 is endogenous vasoactive substance and mitogen. Its effects are mediated by endothelin type A and B receptors. EDN1 is over-expressed in several hypertension models including hypertension rats induced by AngII\[22,23\], EDN1, mainly synthesized by endothelial cells, could also be produced in VSMCs, myocardioocytes and cardiac fibroblasts. In cardiac fibroblasts induced by AngII, reactive oxygen species (ROS) mediated expression of EDN1\[24,25\]. A surprising link between EDN1 and AFs was first suggested in a recent study that cultured mouse aorta AFs expressed ETA and ETB receptors for EDN1, and could synthesize and secret EDN1 in response to AngII. ETA receptor blocker, BQ123 inhibited expression of collagen I α1 at transcription and protein level\[26\]. The role and involved pathways of EDN1 in MF differentiation induced by TGF-β1 are not yet clear. Is it possible related to ROS? Vascular NAD(P)H oxidase is comprised of several subunits, including two transmembrane subunits, p22\(^{phox}\) and gp91\(^{phox}\), and multiple cytoplasmic subunits, p47\(^{phox}\), p67\(^{phox}\) and p40\(^{phox}\), as well as two G proteins, rac and rap1A. Lambeth et al.\[27\] proposed that there was a set of NAD(P)H oxidase family homologized with gp91\(^{phox}\)(NOX2) structurally in vessels and VSMCs expressed NOX1 and NOX4. At present, a great deal of evidences indicate that AngII is able to activate vascular NAD(P)H oxidase and increase ROS production. Adventitia is headstream of superoxide anion production in mice, rats and rabbits. Our previous study showed ROS produced by NAD(P)H increased significantly during the differentiation of AFs to MFs induced by AngII\[28\]. Moreover, it was exhibited that NOX4 mediated transformation from cardiac fibroblasts to myofibroblasts induced by TGF-β1 via activating Smad 2/3, and played an important role in cardiac fibrosis related to heart failure\[29\]. We discovered for the first time that TGF-β1 treatment on AFs resulted in a sustained up-regulation of NOX4 expression, which prompted the probability that ROS may be involved in MF differentiation induced by TGF-β1. Nevertheless, NOX4 is the only gene whose expression profile confirmed by RT-PCR is contradictory to chip results (Fig.3). So the relationship between NOX4 and MF differentiation, as well as whether activation of NOX4 is associated with EDN1 expression need further investigation. Furthermore, MF differentiation induced by AngII or TGF-β1 regulates some molecules in common, such as EDN1 up-regulated by both AngII and TGF-β1, which prompt that MF differentiations induced by AngII and TGF-β1 may share some same pathways. These pathways may be specific for cell phenotypic transformation. Another gene responsible for cell protection in oxidative stress pathway, NQO1, attracts us for its persistent down-regulation in this process. We will give further attention to its relationship with MF differentiation.

Besides these genes with specific function, there are still 890 genes with unknown biological function and EST fragments. They are attractive potential resources for exploring the mechanism of MF differentiation further. Taken together, oligonucleotide microarray results provide abundant molecular information about MF differentiation, which not only confirm parts of present research results, but also widen our knowledge of MF phenotypic transformation via discovering some genes, especially complement elements which respond to TGF-β1 obviously, and give valuable clues for elucidating molecular mechanism of MF differentiation.

REFERENCES


