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Microarray and bioinformatic detection of novel and established genes expressed in experimental anti-Thy1 nephritis

Denise M. Sadlier, Xuesong Ouyang, Blaithin McMahon, Wei Mu, Ryuji Ohashi, Karen Rodgers, David Murray, Takahiko Nakagawa, Catherine Godson, Peter Doran, Hugh R. Brady, and Rick J. Johnson

Department of Medicine and Therapeutics, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Ireland; Mater Misericordiae University Hospital and Dublin Molecular Medicine Centre, Dublin, Ireland; Division of Nephrology, Hypertension and Renal Transplantation, University of Florida, Gainesville, Florida; and Division of Nephrology, Baylor College of Medicine, Houston, Texas

Microarray and bioinformatic detection of novel and established genes expressed in experimental anti-Thy1 nephritis.

Background. Microarray technology is a powerful tool that can probe the molecular pathogenesis of renal injury. In this present study microarray analysis was used to monitor serial changes in the renal transcriptome of a rat model of mesangial proliferative glomerulonephritis. Administration of anti-Thy1 antibody results in phases of acute mesangial injury (day 2), cell proliferation (day 5), matrix expansion (days 5 and 7), and subsequent healing (day 14).

Methods. Using Affymetrix (RAE230A) microarrays coupled with sequential primary biologic function-focused and secondary "baited" global cluster analysis, a cohort of established and putative novel modulators of mesangial cell turnover was identified.

Results. Cluster analysis of proliferative genes identified a number of gene expression profiles. The most striking pattern was increased gene expression at day 5, a cluster that included platelet-derived growth factor (PDGF), cyclins and transforming growth factor-β (TGF-β). The gene expression patterns identified by primary focused cluster analysis were used as bioinformatic bait and resulted in the identification of novel families of genes such as the S100 family. The expression of established and novel genes was confirmed using reverse transcription-polymerase chain reaction (RT-PCR). Next, in vivo gene expression was compared to PDGF-stimulated mesangial cells in vitro revealing similar patterns of dysregulation.

Conclusion. Transcriptomic analysis defined both known and novel molecules involved in mesangial cell proliferation in vitro and in vivo and defined a panel of molecules that are potential contributors to mesangial cell dysfunction in glomerular disease.

Key words: anti-Thy1 nephritis, mesangial cell proliferation, transforming growth factor beta (TGF-β), enigma, platelet-derived growth factor (PDGF), IgA nephropathy (IgAN).

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Mesangial cell proliferation is a key pathogenic factor in a number of major diseases including IgA nephropathy (IgAN) and lupus nephritis [1, 2]. Proliferating mesangial cells undergo a phenotypic switch to an "activated" phenotype and release a variety of mediators with potent local paracrine and autocrine actions that perturb glomerular structure and filtration. In some cases mesangial cell proliferation is associated with dysregulation of extracellular matrix metabolism and mesangial matrix expansion [2]. The molecular triggers for mesangial cell proliferation and the intracellular signaling responses that disturb mesangial cell activation state and function are still being appreciated. While important roles for growth factors such as platelet-derived growth factor (PDGF) have been defined, it is likely that these represent but a fraction of a series of complex and interrelated molecular pathways that together dictate the final glomerular phenotype.

Microarray technology permits large scale profiling of gene expression and is a powerful tool with which to probe the molecular pathogenesis of renal injury. In this study, microarray analysis was used to probe the molecular mechanisms that underpin mesangial cell proliferation. This was done by the exploration of in vivo and in vitro models of mesangial cell proliferation. Initially, microarray analysis was used to monitor serial changes in the renal transcriptome during the evolution of anti-Thy1 nephritis model of mesangial proliferative glomerulonephritis. In this model, administration of anti-Thy1 antibody is associated with phases of acute mesangial injury, cell proliferation, matrix expansion, and subsequent healing with cell proliferation peaking at 5 days post-anti-Thy1 antibody. Using a bioinformatic approach that incorporates sequential primary biologic function–focused and secondary "baited" global cluster analysis, established and putative novel modulators of mesangial cell proliferation were identified. However, to further

explore the role of these modulators on mesangial cell proliferation, the expression of a cohort of these genes was studied in an in vitro model of mesangial cell proliferation. Here, PDGF stimulation resulted mesangial cell proliferation and these cells were subsequently treated with lipoxin, an inhibitor of mesangial cell proliferation.

Simply, using microarray analysis, a cohort of established and novel modulators of mesangial cell proliferation was identified in an in vivo model of mesangial cell proliferation. The expression of these genes was then followed in an in vitro model of mesangial cell proliferation. By this method it was possible to define a panel of molecules that are potential contributors to mesangial cell dysfunction in glomerular disease.

METHODS

Rodent model of anti-Thy1 mesangial proliferative glomerulonephritis in vivo

Wistar rats weighing 180 to 200 g were kept under standard conditions and were randomly allocated to either control or experimental groups. Experimental proliferative nephritis (anti-Thy1 nephritis) was induced by a single intravenous injection of goat anti-Thy1 antibody at a dose of 0.3 mL/100 g. Age-matched controls were injected with the same volume of isotonic saline. Anti-Thy1–treated animals were sacrificed at days 0, 2, 5, 7, and 14 following injection. Cortical regions of the left kidney from each time point were collected, and glomeruli were isolated using the sieving method while right kidneys were collected for histologic analysis.

Human mesangial cell culture in vitro

Human mesangial cells were isolated from a nephrectomy sample obtained from the Mater Misercordiae University Hospital in accordance with institutional, ethical guidelines. As previously described [3], a sample of cortex was isolated and differentially sieved in order to extract the glomeruli, which were subsequently grown on collagen-coated plates. Cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), penicillin (100 U/mL), and streptomycin (100 μg/mL), which was selective for mesangial cell growth. These cells retained the phenotypic characteristics of mesangial cells, including stellate morphology, positive staining for vimentin and alpha-smooth muscle actin (α-SMA) and negative staining for zonula occludens-l antigen (ZO-1) and occluding [1]. Human mesangial cells were serum restricted (RPMI 1640 supplemented with 0.2% FCS) for 48 hours prior to serum starving (RPMI 1640 supplemented with 0% FCS) for a further 1 hour before stimulating. Cells were subsequently treated with PDGF (10 ng/mL) in 0% FCS RPMI 1640 for 24 hours.

Oligonucleotide microarray analysis

RNA isolation, cDNA synthesis, in vitro transcription, and microarray analysis were performed as previously reported [4]. Briefly, total RNA was isolated from whole kidneys at baseline and 2, 5, 7, and 14 days postinjection of anti-Thy1 antibody and from mesangial cells in vitro using RNeasy Mini Column (Qiagen, Valencia, CA, USA). cDNA was synthesized from total RNA using Superscript Choice Kit (Invitrogen, Carlsbad, CA, USA). Biotin-labeled cRNA prepared from template cDNAs was fragmented and hybridized to the Affymetrix RAE230A arrays as per Affymetrix protocol (Affymetrix, Santa Clara, CA, USA). Arrays were then washed and fluorescently labeled prior to scanning with a confocal scanner (Affymetrix). All in vivo time points were microarrayed as experimental duplicates.

With regard to the mesangial cell in vitro experiment, RNA from three independent experiments was pooled after isolation using lysis buffer guanidine isothiocyanate (RLT) and β-mercaptoethanol in accordance with the Qiagen minicolumn preparation (Qiagen, Valencia, CA, USA). Complementary DNA (cDNA) synthesis, in vitro transcription, and microarray analysis were carried out as has been described. As before, all in vitro time points were microarrayed as experimental duplicates.

Bioinformatic and data analysis

Image files were obtained through Affymetrix GeneChip software (MAS5). Subsequently, robust multichip analysis (RMA) was performed [5, 6]. RMA is an R-based technique that analyses directly from the Affymetrix microarray *.*cel image file* and comprises three steps: background adjustment, quantile normalization, and summarization. In order to use a Microsoft Windows operating system RMAexpress was used. This package is a stand-alone graphical user interfaces (GUI) program specifically designed to operate on Microsoft Windows and exclusively performs RMA [5, 6].

For each in vivo time point, an average RMA value was computed for duplicate microarrays and to ensure the average was statistically representative a *t* test and *P* value were generated. Only those genes with a *P* value of ≤ 0.01 were included in subsequent bioinformatic analysis [7, 8]. Thereafter, expression data for each time point were compared to control and a signal log ratio of 0.6 or greater (equivalent to a fold change in expression of 1.5 or greater) was taken to identify significant differential regulation.

Using normalized RMA values, cluster analysis was performed by the program of Eisen et al [9] entitled, "Unsupervised Average Linkage Hierarchical Cluster Analysis." Cluster analysis groups together genes with comparable patterns of expression by employing mathematical methods of similarity organize patterns of