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Pro-inflammatory/Th1 gene expression shift in high glucose stimulated mesangial cells and tubular epithelial cells

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ABSTRACT

Diabetic nephropathy (DN) is a major cause of end stage kidney disease and a strong risk factor for cardiovascular diseases. Growing data show chronic inflammation plays an important role for the progression of DN. As for the immune cells, anti-inflammatory leukocytes as well as pro-inflammatory leukocytes play an important role in DN. In addition to leukocytes, renal resident cells contribute to the inflammatory process of DN. However, precise functions, phenotypes and immune balance of renal resident cells remain to be explored. Therefore, we hypothesized that the aberrant immune balance of renal resident cells contributes to the pathogenesis of DN. To explore this possibility, we performed genome-wide transcriptome profiling in mesangial cells and tubular epithelial cells (TECs), which were stimulated by high glucose (HG) and detected the expression of inflammation associated genes. HG increased the mRNA expression of oxidative stress, inflammasome and mammalian target of rapamycin (mTOR) related genes in mesangial cells. Pro-inflammatory/Th1 gene expression was upregulated, but Th2 related gene expression was downregulated in mesangial cells. In TECs, HG stimulation increased pro-inflammatory/Th1/Th2 gene expression. Phosphorylation of signaling proteins shifted towards pro-inflammatory phenotype with suppressed phosphorylation of Th2 related signaling in TECs. The data taken together indicate that HG shifts the immune balance toward pro-inflammatory/Th1 phenotype in mesangial cells and TECs, which might initiate and/or prolong inflammation, thereby resulting in diabetic nephropathy.

1. Introduction

The number of patients with end stage kidney diseases (ESKD) is increasing worldwide. Especially, diabetic nephropathy (DN) is one of the major causes of ESKD in developed countries. Not only the high incidence of ESKD, but also the high mortality from cardiovascular diseases has been observed in patients with DN [1]. Therefore, identifying molecular mechanisms that mediate the progression of DN is an important issue to uncover novel therapeutic targets.

Growing evidence suggests that chronic inflammation plays key roles for the progression of DN [2–4]. Recent studies revealed that macrophages (Mϕ) and T cells contribute to the pathogenesis of DN in both rodent and human models. Activated Mϕ and T cells induce and aggravate the kidney injury, resulting in chronic kidney failure. Recently, Mϕ are categorized as inflammatory (M1) and anti-inflammatory (M2) phenotype according to the cytokine profile and cell surface marker [5]. M2 Mϕ as well as M1 Mϕ has been reported to contribute to the pathogenesis of DN [6–8]. $CD4^+$ T cell populations are also broadly and simplistically divided into 4 types, Th1, Th2, Th17 and regulatory T cells based on the function [9]. Each phenotype of Th cell has been reported as a key player for the progression of DN [10–12]. These data suggest that the immune balance of inflammatory cells affect the prognosis of DN.

Not only accumulated leukocytes, but also renal resident cells orchestrate in the inflammatory processes in DN. Activated renal resident cells secret various cytokines/chemokines [13,14]. However, precise functions, phenotypes and immune balance of renal resident cells remain to be explored in DN. Therefore, we hypothesized that the aberrant immune balance of renal resident cells

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may contribute to the pathogenesis of DM. To explore this hypothesis, we performed genome-wide transcriptome profiling in mesangial cells and tubular epithelial cells (TECs), which were stimulated by high glucose (HG), and identified the expression of inflammatory associated genes.

2. Materials and methods

2.1. Cell culture

Normal human mesangial cells (Lonza, Basel, Switzerland) were cultured in mesangial culture kit (Lonza). Human immortalized TECs, HK-2 (ATCC, Manassas, VA) was cultured in 10% FBS DMEM (Life technologies, Tokyo) with 100 μg/ml streptomycin and 100 U/ml penicillin. All the cells were grown in a humidified atmosphere (5% CO^2/95% air) at 37 °C and were seeded onto six-well cell culture cluster (Corning Incorporated, Corning, NY). The cells were stimulated with 30 mM glucose (Wako, Tokyo) for 24 h for hyperglycemic stimulation. For osmotic control, the cells were cultured with 30 mM mannitol (Wako) for 24 h.

2.2. SAGE analysis using Ion Torrent PGM™

Gene expression analysis was performed by SAGE method and NGS. Briefly, RNA was isolated using mRNA Isolation kit (Sigma–Aldrich, St. louis, MO). NGS data from the PGM™ was generated from 1 μg of total RNA isolated from cell line. SAGE libraries were constructed using the SOLiD SAGE™ kit from Life Technologies (Life Technologies) according to manufacturer's protocol. DNA was recovered from the agarose gel using PureLink Gel Extraction kit (Life Technologies). DNA fragments of SAGE construct were analyzed on the Agilent Bioanalyzer using the High Sensitivity Kit (Agilent, Santa Clara, CA). Template preparation and emulsion PCR, and Ion Sphere Particles (ISP) enrichment was done using the Ion Xpress Template kit (Life Technologies) according to the manufacturer's instructions. The quality of the resultant ISPs was assessed using Qubit 2.0 Fluorometer (Life Technologies), and were loaded and sequenced on a 318 chip (Life Technologies). The complete data sets from these experiments have been deposited in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo; accession number GSE52734).

2.3. Mapping from NGS data

For each of the samples, the PGM™ raw reads were aligned against the human refseq genes (UCSC, http/hgdownload.cse.ucsc.edu/) using BWA 0.6.2 which uses the 25_1 mapping parameter. We generated unique gene counts by excluding reads that mapped to contigs of more than one gene. Reads mapping to several contigs within an isogroup were only counted once.

2.4. Antibodies

The following antibodies were used for flow cytometric analysis; Phycoerythrin (PE) conjugated anti-phosphorylated p38 (612565; BD Biosciences, San Jose, CA), PE-conjugated anti-phosphorylated STAT1 (562674; BD Biosciences), PE-conjugated anti-phosphorylated STAT3 (558557; BD Biosciences), PE-conjugated anti-phosphorylated STAT5 (61257; BD Biosciences), PE-conjugated anti-phosphorylated STAT6 (612701; BD Biosciences).

2.5. Flow cytometric analysis

To detect phosphorylated intracellular signaling, cells were fixed and permeabilized using BD Phosflow™ in accordance with the manufacturer's protocol (BD Biosciences). Then, the cells were stained with antibody. We collected 10,000 cultured cells using FACSCalibur (BD Biosciences) and analyzed data using FlowJo software 9.3 (Tree Star, Palo Alto, CA).

2.6. Reconstitution of signal transduction pathway

To identify the signal transduction pathway, each gene was reconstituted according to Kyoto Encyclopedia of Genes and Genomes (KEGG: http://www.genome.jp/kegg/) database.

2.7. Statistics

Statistical analysis was performed as described before [15]. Data represent the expression ratio (HG stimulated/osmotic control).

3. Result

3.1. High glucose (HG) increased the mRNA expression of oxidative stress, inflammasome and mammalian target of rapamycin (mTOR) related genes

Oxidative stress [16,17] has been regarded as intracellular mediator in HG induced inflammation. Thus, we examined the mRNA expression of oxidative stress associated genes. Among them, reactive oxygen species modulater 1 (ROMO) and protein kinase C (PKC) alpha (PRKCA) mRNA were increased in HG stimulated mesangial cells (Fig. 1). Recent study revealed that PKC signaling is closely related to mTOR signaling [18,19]. Therefore, we analyzed mTOR signaling related genes. Interestingly, the relative gene expression of mTOR and its downstream signaling, translation initiation factor 4B (EIF4B) were upregulated. Moreover, thioredoxin interacting protein (TXNIP) mRNA, which has the potential to activate the NOD-like receptor family, pyrin domain containing (NLRP) 3 inflammasome [20], was also increased in HG stimulated mesangial cell. In addition, transcription factor, activated protein (AP)-1 (JUN, FOSL2), C–C chemokine ligand (CCL) 2 and tumor necrosis factor (TNF)-α mRNA were also increased higher in HG stimulated mesangial cells as compared to osmotic control.

3.2. HG stimulated mesangial cells were skewed toward an inflammatory phenotype

As HG increased the mRNA expression levels of inflammatory signal mediators, we look into whether HG stimulation shifted the cell phenotype toward an inflammatory population. Inflammatory cytokines/chemokines and their receptors, such as CCL2, TNF-α and interleukin(IL)-1 receptor mRNA expression were increased by the HG stimulation in mesangial cells. Moreover, mitogen activated protein kinase (MAPK) mRNA expression was also increased in HG stimulated mesangial cells. In contrast, Th2 related cytokine receptors and intracellular signaling molecules showed the reduced mRNA expression levels (Table 1). Taken together, HG stimulation increased mRNA expression of the pro-inflammatory molecules and decreased Th2 related molecules in mesangial cells.

3.3. Increased mRNA expression of inflammatory cytokines/chemokines and Th2 type cytokine receptors in HG stimulated TECs

Next, we examined the functional phenotype of HG stimulated TECs. Stimulated TECs showed the increased mRMA levels of inflammatory cytokines/chemokines and their receptors, such as TNF-α, IL-1 receptor, IL-18, IL-12 and IL-6. In addition, mRNA expression of Th2 related cytokine receptors, IL-10 receptor and IL-13 receptor, also increased via HG stimulation (Table. 2).