Quantitative gene expression of TGF-β1, IL-10, TNF-α and Fas Ligand in renal cortex and medulla

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Abstract

Background. Inflammatory, fibrogenic or apoptotic processes in the kidney are regulated by intra- and intercellular mediators. Intrarenal upregulation of genes may precede structural changes by days and can be examined in extremely small amounts of tissue. With the advent of new quantitative PCR methods results of gene expression are available within few hours after kidney biopsies.

Methods. In order to establish reference values for intrarenal gene expression of TGF-β1, IL-10, TNF-α and Fas Ligand in renal cortex and medulla, we analysed 28 histologically normal kidney samples available after tumour nephrectomy by quantitative real-time PCR. After reverse transcription of isolated RNA, cDNA aliquots were quantified for target genes using the threshold cycle (Ct) method normalized for the house keeping gene GAPDH.

Results. Expression of target genes was lower in cortex as compared to medulla, but the differences were only significant for IL-10 (P=0.0125). TGF-β1 was found with the highest gene expression about five PCR-cycles (delta Ct) after GAPDH with markedly lower results for TNF-α (delta Ct ~ 9), IL-10 (~ 12) and Fas Ligand (~ 14).

Conclusions. These results are the first reported reference values for human intrarenal gene expression which should facilitate interpretation of data from native or transplant kidneys in future studies.

Keywords: cytokines; delta delta CT method; intrarenal gene expression; kidney transplantation; quantitative RT-PCR

Introduction

With the development of quantitative PCR methods intrarenal gene expression has been increasingly studied in native kidneys [1–2] and transplant biopsies [3–4]. Gene expression data are usually correlated to clinical course and biopsy histology and offer potential insight into the cellular programme of the underlying disease. Intrarenal upregulation of gene expression may precede structural alterations by days [5]. In addition, only small amounts of tissue or cells are required for analysis of gene expression and, therefore, fine needle aspirates may offer a tool for sequential studies during an acute or chronic process. Results are available within few hours after renal biopsy.

A huge number of intra- and intercellular mediators are of importance in regulating inflammatory, fibrogenic or apoptotic processes in the kidney. We quantitated gene expression rates of tumour necrosis factor-α (TNF-α), interleukin-10 (IL-10), transforming growth factor-β1 (TGF-β1) and Fas Ligand (FasL) by normalization for transcription rates of the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [6]. These target genes were chosen because they have been frequently studied in native renal diseases and particularly after kidney transplantation [3,4,7,8]. Special attention has been focused on a variety of polymorphisms in the regulatory regions of these target genes and possible clinical consequences [9,10].

We thus analysed gene expression in cortex and medulla to obtain reference values which we believe are of great importance for other researchers and clinicians studying renal disease and allograft rejection, especially since with the advent of real-time PCR and premixed standardized reaction reagents inter-laboratory comparison becomes feasible.
Material and methods

Nephrectomy specimens from surgeries due to renal cell carcinoma were available in 28 patients (12 female, 16 male) with a mean age of 57.4 years (SD 17 years). Sections of macroscopically tumour-free kidney were used to separate cortex and medulla, and samples were snap frozen in liquid nitrogen prior to storage at −70°C for subsequent RNA extraction. Sections from the immediate vicinity of these samples were taken for routine histology, stained and reviewed for absence of neoplasma or inflammation as published previously [11]. Total RNA was extracted after homogenization with a Polytron in 4M guanidinium-isothiocyanate according to a standard cesium chloride method [12]. Purity and yield was photometrically determined and 1 µg of total RNA was reverse transcribed by Maloney murine leukaemia virus transcriptase in a final volume of 40 µl as described previously [13].

All quantitative real-time PCR (TaqMan™) primers and probes were obtained from Applied Biosystems (Weiterstadt, Germany). For detection of GAPDH, IL-10, TNF-α and TGF-β1 pre-developed assay reagents (PDAR) were available. Primers and probe for Fas Ligand (Table 1) were designed to span from exon 3 to 4.

All PCRs were performed utilizing 0.2 µl cDNA per reaction in triplicates of 25 µl volume on a ABI Prism 7700 Sequence Detection System (TaqMan) using a 2-step PCR protocol after the initial denaturing of the cDNA (10 min at 95°C) with 45 cycles of 95°C for 15 s and 60°C for 1 min. Universal master mix as obtained from Applied Biosystems included all reagents including Taq-polymerase apart from specific primers and probes.

Dilution experiments were performed to ensure similar efficiency of the PCRs, and standard curves were calculated referring the threshold cycle (Ct, the PCR cycle at which a specific fluorescence becomes detectable) to the log of each cDNA dilution step [6,14].

All amplification batches included no template controls (NTC) and cDNA aliquots from an acutely rejected renal cDNA dilution step [6,14]. Specific fluorescence becomes detectable to the log of each target with gradients ranging from −3.3 to −3.58 (R² 0.924 to 0.999, Figure 2). Thus, results of cytokines were normalized to GAPDH by calculating the differences in Ct. Over a period of 6 months, aliquots of a cDNA positive control were repeatedly quantified in triplicates under standard conditions with a mean detection of GAPDH at Ct 31.2 (n = 19, SE ± 0.058 Ct, intra-assay variability 0.10, inter-assay variability 0.37). Real-time PCR resulted in the lowest Ct and thus strongest expression for GAPDH in medulla (mean 21.8; SE ± 0.16) and cortex (21.6 ± 0.17) followed by TGF-β1 (medulla: 26.5 ± 0.47; cortex: 27.2 ± 0.53) as illustrated in Figure 3. IL-10 was detected at mean Ct 33.6 ± 0.31 in medulla and 35.0 ± 0.36 in cortex, TNF-α at 31.3 ± 0.37 in medulla and 31.8 ± 0.40 in cortex and FasL at 35.4 ± 0.24 in medulla and 35.8 ± 0.31 in cortex. Significant differences between cortex and medulla were only found for IL-10 (P ≤ 0.0125, paired sign test). The Ct-threshold was set at 0.02 for detection of TGF-β1, TNF-α and GAPDH and at 0.03 for IL-10 and FasL in all PCRs.

Next, we analysed individually for each kidney the difference of target gene detection related to GAPDH in cortex and medulla by the so-called delta delta Ct-method [19]. Although a tendency for lower expression of target genes as normalized for GAPDH was found for cortex samples as compared to medulla (Figure 4), this difference was again only significant for IL-10 (P = 0.0125, paired sign test). Variation and interquartile ranges of target gene expression normalized to GAPDH are presented in Table 2.

Reproducibility of results was demonstrated by analysing 36 cDNA samples for GAPDH and TGF-β1 in Edmonton, Canada and Hannover, Germany as depicted in Fig. 5. No significant difference was noted (paired t-test: P = 0.34, mean difference 0.147, 95% confidence interval between −0.159 and 0.452).

Results

Real-time PCR of GAPDH, IL-10, TNF-α, TGF-β1 and FasL showed a rapid and consistent amplification of cDNA in all samples obtained from normal renal cortex and medulla. Neither negative controls nor genomic DNA lead to elevated fluorescence signals after PCR (Figure 1).

Electrophoresis of PCR-products and ethidium bromide staining was only performed during method evaluation and revealed no bands from NTCs and single bands for all targets in cDNA PCR products (size 80 to 150 bp). Single bands of longer size were also detectable in genomic PCR products of IL-10, TGF-β1 and FasL but did not result in raised fluorescence signals in real-time PCR.

Dilution experiments of cDNA aliquots resulted in a very similar amplification efficiency of the different targets with gradients ranging from −3.3 to −3.58 (R² 0.924 to 0.999, Figure 2). Thus, results of cytokines were normalized to GAPDH by calculating the differences in Ct. Over a period of 6 months, aliquots of a cDNA positive control were repeatedly quantified in triplicates under standard conditions with a mean detection of GAPDH at Ct 31.2 (n = 19, SE ± 0.058 Ct, intra-assay variability 0.10, inter-assay variability 0.37).

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Table 1. Sequence of primer-probe combination to quantify human Fas Ligand

<table>
<thead>
<tr>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Dual-labelled fluorescent probe</th>
<th>PCR-product</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAAGTGCCCCATTTAAACGGC</td>
<td>AAAGCAGGCAATTCCATAGTTG</td>
<td>FAM-TCCAACCTAAAGGTCCATGCTCGTGG-TAMRA</td>
<td>82 bp only in cDNA</td>
</tr>
</tbody>
</table>
Discussion

Our study for the first time demonstrates that a baseline transcription of the proinflammatory cytokines TGF-β1 and TNF-α, IL-10 and the proapoptotic FasL gene occurs in native undiseased kidney and is readily and always detectable by Taqman™ based real-time PCR in both medulla and cortex within narrow ranges. Compared to GAPDH, very little intrarenal gene activation of all target genes except
for TGF-β1 was found (Figure 3). Although there was a trend towards higher gene expression in medulla as compared to cortex, these differences were only significant for IL-10 ($P = 0.0125$, paired sign test). A predominant involvement of gene expression in the medulla as compared to cortex was also observed by other groups in experimental nephropathy [15].

Histologic evaluation of the kidney sections in the direct vicinity of the tissue chosen for RNA extraction had not revealed any cellular infiltrates and other pathologic alterations not attributed to normal ageing [11]. Thus, transcription rates of our target genes are unlikely to be caused by extrarenal cells invading the tissue. Exact location and type of cells responsible for gene expression cannot be identified by our approach. This topic may be addressed by using non-RNA-denaturing microdissection techniques [16], which may offer a chance to differentiate between interstitium, vascular structures and glomeruli. However, this technique is not yet generally available. Neither did we investigate post-transcriptional regulation of target genes or problems of tissue sampling in focal disease. However, others and we have demonstrated the diagnostic options of measuring gene expression on RNA-level which can easily be performed in small (2 mm) sections of a normal biopsy cylinders [3,4,17].

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**Fig. 2.** Standard curves for detection of target genes: diluted cDNA aliquots vs threshold cycle ($C_t$). Similar gradient means similar PCR efficiency for the targets investigated.

**Fig. 3.** Threshold cycle ($C_t$) of 28 renal medulla and cortex cDNA aliquots amplified for different target genes. Depicted are 10th, 25th, 50th, 75th and 90th percentile. Significant differences were only detected for IL-10 ($P = 0.0125$, paired sign test).
Rigid comparison of competitive template PCR and Taqman™ based real-time PCR has revealed a similar sensitivity and reproducibility in minimal residual disease in leukaemia [6]. As little as ten target gene copies can be reliably quantified by either technique. However, real-time PCR is very readily available, gives immediate information on cycle to cycle generation of PCR products and eliminates the need to exchange and verify standards when utilizing the delta delta Ct-method. The approach was chosen after eliminating genomic DNA interference by cDNA-specific primer–probe combinations and ensuring an almost identical PCR efficacy for the target genes studied. Provided technical comparability of RNA-isolation, reverse transcription and reagents is assured, our data may provide a first guideline to assess both augmentation and downregulation of gene transcription in native kidney disease and transplantation. The method has acceptable intra and inter assay variability, especially considering its sensitivity. The standard error of 0.058 Ct observed in our real-time PCR over a period of 6 months corresponds to 0.02–0.18 reported by other investigators [14]. We were able to demonstrate that this technique is appropriate for inter-laboratory comparative work with differences in the range of normal variation of the method.

As most biopsies are performed in diseased or transplanted kidneys and gene expression studies require rapid RNA protection, suited control tissue samples are not easily available accounting for the limited number of 28 kidneys examined in this study. As of now it cannot be judged if low gene expression in renal tissue is subnormal or averages the transcription rates in normal tissue. Rather than estimating x-fold increases of gene expression, we present raw data and differences in Ct between target gene and GAPDH for better comparison with other investigators. Expression of most target genes of this study may be regulated by the presence of polymorphisms in or near the promoter region of the respective genes [10]. Various studies have been published relating certain genotypes to an increased risk for organ rejection [9] or fibrosis [18]. However, data on the direct influence of these polymorphisms on gene expression in vivo are scarce and their clinical value has recently been doubted [19]. In renal allografts we did not detect an association

**Table 2.** Quantification results of target genes by real-time PCR: means, standard errors, medians and interquartile ranges as normalized for GAPDH by the delta delta Ct method

<table>
<thead>
<tr>
<th></th>
<th>IL-10</th>
<th>TGF-β1</th>
<th>TNF-α</th>
<th>FasL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>medulla</td>
<td>cortex</td>
<td>medulla</td>
<td>cortex</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>-11.8 ± 0.30</td>
<td>-13.5 ± 0.34</td>
<td>-4.7 ± 0.40</td>
<td>-5.55 ± 0.51</td>
</tr>
<tr>
<td>Median</td>
<td>-11.9</td>
<td>-13.6</td>
<td>-4.1</td>
<td>-4.9</td>
</tr>
<tr>
<td>25% quartile</td>
<td>-12.9</td>
<td>-14.7</td>
<td>-4.8</td>
<td>-6.3</td>
</tr>
<tr>
<td>75% quartile</td>
<td>-10.8</td>
<td>-12.5</td>
<td>-3.6</td>
<td>-4.0</td>
</tr>
</tbody>
</table>

**Fig. 4.** Gene expression of target genes normalized for GAPDH by the differences in threshold cycle (Ct) in 28 renal medulla and cortex cDNA aliquots. Depicted are 10th, 25th, 50th, 75th and 90th percentile. Significant differences were only detected for IL-10 (P=0.0125, paired sign test).
of certain polymorphisms proposed as high-, inter-
mEDIATE- or low producer genotypes with intrarenal 
expression of these cytokines [20].

We conclude that direct interlaboratory comparison 
of our intrarenal transcription data on GAPDH, 
IL-10, TNF-a, TGF-b1 and FasL is feasible due to 
the highly standardized reaction conditions used in 
this study and pre-mixed PCR reagents commercially 
available from various companies. Thus, we believe 
that the reported expression rates of these targets genes 
may serve as an important baseline reference for other 
scientists investigating intrarenal gene expression in 
native or transplant kidneys.

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Fig. 5. Inter-laboratory correlation of quantitative real-time PCR. Regression curve and line of identity ± 1 C_t (dotted) are depicted. Differences between results from Hannover, Germany and Edmonton, Canada were not significant (paired t-test: P = 0.34).
Cytokine gene expression in renal cortex and medulla


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