A novel evaluation method for paraffinized human renal biopsies using quantitative analysis of microdissected glomeruli and VCAM-1 as marker of inflammatory mesangial cell activation

Jochen W. U. Fries1, Tanja Roth1, Hans-Peter Dienes1, Manfred Weber2 and Margarete Odenthal1

1Department of Pathology, University of Cologne and 2Department of Internal Medicine I, Cologne General Hospital, Merheim Medical Center, Cologne, Germany

Abstract

Background. In the glomerular mesangium, immunologic and/or infectious activation of the inflammatory, NF-κB-mediated signal pathway can induce a progression of already existing mesangial lesions in non-immunologic and immunologic glomerular disease. This progression is preceded by upregulated mesangial gene expression of which the vascular cell adhesion molecule-1, VCAM-1 (vascular cell adhesion molecule-1), is a well-established marker. Its evaluation on minimal tissue such as routinely paraffinized needle core biopsies is not established and needs the development of a novel evaluation method more meaningful than common immunohistology.

Methods. By laser-microdissection, 10 glomeruli/case were isolated from 5 μm thick tissue slices in a total of 15 cases of mesangial proliferation with different renal diseases (IgA nephropathy, lupus nephritis and mesangial proliferative lesions of unknown aetiology) vs transplant biopsies as negative and TNF α-treated cultured human mesangial cells as positive controls. After reverse transcription of isolated RNA, cDNA aliquots were quantified for VCAM-1 expression by real-time PCR using the threshold cycle (Ct) method, normalized for the housekeeping gene β-actin, and compared with qualitative RT–PCR results.

Results. Unsuspected VCAM-1 transcript steady-state levels could be detected by real-time PCR in agreement with qualitative PCR, while morphologic and immunohistologic analyses were unrevealing. As yields of RNA extraction in femtogram quantities cannot be measured spectrophotometrically, a Ct-ratio was formed between β-actin and VCAM-1 per case showing high VCAM-1 expression in lupus nephritis (1.39), and moderate expression in IgA nephropathy (1.08–1.23) vs TNF α-treated mesangial cells (0.97–1.23) and negative control cases (0.66–0.68).

Conclusions. This is the first reported gene expression analysis method for routinely paraffinized human renal biopsies, demonstrating the power of combined laser-microdissection and PCR quantification as novel methods for the evaluation of minimal tissue beyond purely descriptive morphologic analysis.

Keywords: human; kidney; laser-microdissected glomeruli; paraffinized biopsies; quantitative RT–PCR; VCAM-1

Introduction

In the pathogenesis of mesangial proliferative renal lesions, many genes may play an important role. Their predictive value for diagnosis and possible therapeutic use, however, remains to be established. For the last 25 years, classic pathologic analyses of human renal biopsies employ an immunologic approach to detect immunoglobulins and complement fractions in the glomerulus using sections of the entire biopsy. In contrast, comparative gene expression analysis between glomeruli of different biopsies with distinct morphologic lesions, however, necessitates a specific isolation of the target structure as well as quantification of the levels of the genes of interest.

Recently, laser-microdissection has been established as a reliable method for procuring morphologically distinct tissue regions of interest in a large variety of tissues. For the kidney, studies have used unfixed, frozen kidney slices from murine models [1–3] providing sufficient tissue to analyse. So far, only a single report exists on the use of one frozen human biopsy [3]. However, routine evaluation of renal biopsies in Europe has been performed for many years on formalin-fixed, paraffinized needle core biopsies.
because of their vast superior preservation of morphologic details compared with frozen material. Thus, presently no study exists describing the specific isolation and RNA extraction from small tissue targets such as glomeruli in routinely fixed and paraffin-embedded needle core biopsies.

As for the second prerequisite, the determination of different levels of gene expression, quantitative PCR methods have been developed. They have been used to study intrarenal gene expression in native kidneys from murine models [4,5], whole organ nephrectomies [6] and larger fractions of frozen human renal core biopsies from transplant kidneys [7]. However, no data presently exist applying this method to minute targets such as glomerular slices after laser-microdissection of formalin-fixed, paraffinized renal biopsies.

Experimental reports have demonstrated the progression of an existing mesangial proliferation due to an immunologic or non-immunologic activation of nuclear-factor kappa B (NF-κB)-mediated inflammatory signal pathway. Our group has shown that activation of this pathway in vitro [8] and in vivo [9] leads to a mesangial surface expression of vascular cell adhesion molecule-1 (VCAM-1), a member of the immunoglobulin supergene family [10].

As intrarenal upregulation of gene expression may proceed structurally detectable alterations by days [11], quantification of VCAM-1 as marker gene for mesangial cell (MC) stimulation may be an important alarm signal indicating further progression of MC lesions. Thus, we describe here a novel evaluation method applicable for routine renal biopsies. It consists of combining laser-microdissection of glomeruli from formalin-fixed, paraffinized renal needle core biopsies with so-called real-time, quantitative RT–PCR, using as study objective the analysis of VCAM-1 expression in mesangial proliferative lesions. As VCAM-1 expression could be detected in mesangial lesions unsuspected of inflammatory activation by standard routine analysis, the specific evaluation of VCAM-1 levels may be important for further diagnostic considerations and possible therapeutic interventions.

**Subjects and methods**

**Tissue and cell procurement**

Archival human renal biopsies, routinely fixed in 4% buffered formalin and paraffin-embedded overnight were pre-analysed by light- and transmission electron microscopy as well as immune histology using antibodies for immunoglobulin heavy chains (IgG, A, M), complement factors (C1, C3, C4) and fibrinogen. Diagnoses were rendered by two independent renal pathologists (J.W.U.F. and H.-P.D.) according to standard criteria. Three cases of lupus nephritis (type IV, diffuse proliferative: subendothelial and mesangial immune complexes positive for IgG, IgM, C1, C3, C4), three cases of IgA nephropathy (mesangial immune deposits positive for IgA and C3), of which two were re-biopsied and two of transplant kidneys at day of transplantation (‘Null biopsies’ as negative controls; negative by immunohistology) as well as five cases of mesangial proliferative lesions with undetermined aetiology and no detectable immune complex deposition were used (Figure 1).

All reagents were purchased in Germany unless otherwise noted. Human mesangial cells were obtained from CellSystems (Biotechnologie Vertrieb GmbH, St Katharinen, Germany) and cultured in MsGM media, containing 10% fetal bovine serum. Subcultures six to eight were used and after serum starvation for 24 h stimulated with media containing either TNF α (1000 U/ml) for 6 h or PDGF BB (10 ng/ml) for 14 h or no stimulation for 24 h.

From a freshly explanted human kidney (with a small urethelial carcinoma and no other detectable parenchymal disease) sent for pathological evaluation by frozen section, a small slice of cortex was immediately frozen in OCT compound (Sakura Finetek Europe B.V., The Netherlands [9]).

**Laser-manipulated microdissection, and laser pressure catapulting**

Ten to 25 glomeruli were individually microdissected from 5 μm thick sections of either mouse kidney or human renal biopsy cores using a laser microbeam (P.A.L.M., Wolfrathshausen, Germany). Contamination by parietal epithelial cells, expressing VCAM-1 constitutively [12], was carefully avoided. Subsequently to dissection, each glom¬erulus was laser-catapulted into a microfuge tube containing RNase-free water, as well as RNAsin (20–40 U/μl, Promega, Mannheim, Germany), and stored frozen at −70°C until extraction.

**RNA extraction and reverse transcription**

RNA was extracted using the Purescript RNA isolation kit (Genta, Minneapolis, MN) according to manufacturer’s instructions with the following modifications and volume adjustments for human renal biopsies. For each micro-dissected sample of 20 glomeruli collected from 5 μm thick formalin-fixed and paraffinized sections, only 30 μl of lysis buffer were used and the tube gently shaken. One microgram of poly A-RNA (Qiagen, Hilden) and 10 μl DNA–protein-precipitation solution were added for 5 min on ice, and centrifuged to form a tight pellet as advised by the manufacturer. The supernate was transferred to a new tube and mixed with 30 μl of 100% ice-cold isopropanol and 1 μl of 2 μg/ml glycogen (Boehringer Ingelheim; no 901393) by inverting 50 times. After centrifugation and washes with 70% ice-cold ethanol a small pellet should be visible which was air dried and resuspended in RNase-free water. Subsequently, an additional DNase (Sigma-Aldrich, Taukirchen) treatment according to manufacturer’s recommendation was performed to prevent amplification from contaminating DNA. These optimized and adjusted volume requirements were tested using batches of microdissected, formalin-fixed and paraffinized mouse glomeruli. Extraction and reverse transcription procedures were also optimized with cDNA from micro-dissected mouse glomeruli. To evaluate the efficiency of this extraction procedure, one of the most recent commercially available extraction kits, called PicoPure RNA isolation kit (Arcturus, Mountain View, CA) was used. According to the manufacturer, this extraction procedure is fast (total extraction time 2 h) and optimized to work on minute tissue samples with as little as 10 microdissected cells. The provided protocol was followed strictly without any additional volume or procedure adjustment. Mouse glomeruli (in separate sets of 20) were microdissected from frozen and
formalin-fixed, paraffinized kidneys and extracted using this protocol. For quantitative analysis, β-actin was measured using the QuantiTect SYBR Green PCR kit (Qiagen, Hilden) with the ABI PRISM 7700 PCR cycler (PE Biosystems, Weiterstadt) in a two-step RT–PCR procedure according to a protocol provided by the manufacturer.

The entire amount of extracted RNA was subsequently used in a reverse transcription reaction (TaqMan reverse transcription reagent kit; PE Biosystems) using random hexamers. cDNA was stored at 4°C until further use.

**Qualitative semi-nested PCR**

For qualitative assessment of VCAM-1 expression, the cDNA material of one to five glomeruli per case was used in a so-called nested PCR reaction. Reverse transcribed RNA from TNF-α-stimulated human mesangial cells served as control. First and second round PCR primers were used as described previously [13]; their products were checked by sequence analysis [13]. Primers for β-actin semi-nested PCR were manufactured by MWG Biotech (Ebersberg, Germany; forward primer: 5'-TTGCAATGAGCGGTTCCGCT-3'; reverse primer 1: 5'-TACAGCTGTTTGGCGATGTCC-3'; reverse primer 2: 5'-CACGTCACACTCATGATGGAG-3'). Each PCR was performed in a total volume of 50 μl at an annealing temperature of 55°C with each run consisting of 30 cycles [13]. All PCR products were analysed using ethidium bromide stained, 1.8% agarose gels.

**Quantitative real-time TaqMan PCR**

For VCAM-1, all primers and the probe were obtained from PE Biosystems. Quantification was performed using the same forward and reverse primers (no. 2) as in the qualitative semi-nested PCR. The probe was 5'-TTCTCTCTGAGCTTCTCCTCTCTTTTGC-3' with its 5' nucleotide labelled with a quencher dye (TAMRA = 6-carboxy-tetramethylrhodamine) and its 3' nucleotide labelled with the fluorescent 6-FAM. For β-actin detection, primers and probe were obtained from Eurogentics (Seraing, Belgium). Universal master mix (PE Biosystems) contained all reagents including Taq-Polymerase apart from specific primers and probes. Volume equivalents of one, three or five glomeruli were tested in a total reaction volume of 50 μl. PCR conditions were as follows: 2 min at 50°C for uracil-N-glycosylase incubation, 10 min at 95°C for the activation of AmpliTaq Gold DNA polymerase and then 60 cycles of 95°C for 15 s and 60°C for 1 min. The expression of VCAM-1 and β-actin in each sample were quantified in separate tubes with the respective primers. The amplification was performed using the ABI 7700 PRISM PCR cycler (PE Biosystems) in a two-step RT–PCR procedure. Gels of the final products were analysed as described before.

**Results**

The present availability of several RNA extraction kits within the last year prompted us to compare one of this new generation to our established extraction procedure. We tested two individual microdissected sets of mouse glomeruli each from frozen vs formalin-fixed, paraffinized renal tissue by quantitative RT–PCR. The Purescript kit (Gentra systems, MN) yielded as
threshold values for β-actin 22.1 and 21.7, the Arcturus kit 31.6 and 30.7 for the frozen samples. For the paraffinized glomeruli, values of 41.5 vs 44.5, respectively, were achieved (data not shown). By serial dilution analysis of β-actin, this difference in cycle numbers translates into an ~1000-fold difference in RNA amount to be detected in frozen vs fixed glomeruli.

In order to validate our Purescript extraction method further, we performed a qualitative RT–PCR using the mRNA equivalent of one glomerular slice (Figure 2). For VCAM-1 a 103 bp amplification product, for β-actin as housekeeping gene, a 110 bp product was obtained.

Using either three paraffinized or two freshly frozen cortical slices from the explanted human kidney, we found a successful and reproducible amplification from the paraffin tissue blocks for the cDNA equivalent of three and five glomerular slices. However, using frozen tissue as glomerular source, the same amplification procedure shows equally reproducible results, but with an increase of RNA yield between 25 and 40% compared with paraffinized glomeruli (Figure 3).

For quantitative real-time PCR (Figure 4) of VCAM-1, the same primer pair was used as for the first set of semi-nested, qualitative PCR to improve comparability between both procedures. Principally, VCAM-1 amplification could be shown in the same cases as in the qualitative RT–PCR evaluation in Figure 2. Three different groups of amplification curves could be differentiated with respect to their CT value and logarithmic increase. Low threshold cycles and steep logarithmic increases were observed in cultured mesangial cells (no. 1), while maximal cycle numbers (=60) with almost flat curve shapes resulted from negative cases (nos 5–7). Positive amplification curves from human biopsies were obtained from biopsies with lupus nephritis (no. 2) and IgA nephropathy (nos 3 and 4). These cases showed characteristic S-shaped amplification curves, however, displayed rather high threshold values, corresponding to low starting material. Thus, using a standard curve for the detection of VCAM-1 depicting serially diluted cDNA samples vs threshold cycles, we found CT values in positive biopsies equivalent to femtograms of cDNA. This result is in contrast to 100 ng of cDNA for cultured mesangial cell samples (data not shown). No detectable fluorescent signals were obtained in negative controls.
controls (no template, no Taq enzyme). The two mesangial proliferative lesions of unknown etiology, positive for VCAM-1 in qualitative PCR, did not yield enough glomeruli for quantitative analysis, indicating that the remaining material from minute biopsies was insufficient for this procedure. Electrophoresis of selected final PCR products displayed amplification products of the expected size (103 bp, data not shown).

To correct for the differences in mRNA loading for PCR amplifications, and thus to enable comparability of the observed values, amplification curves for β-actin as control were performed for each sample tested as for VCAM-1 and a ratio between the C_T values of β-actin and VCAM-1 was calculated (Table 1). In addition, a second set of biopsy cases was analysed by quantitative PCR including also a second biopsy in two cases of IgA nephropathy. Table 1 shows data from all materials studied by real-time PCR. Comparable expression values are shown from different sets of mesangial cells as well as from all biopsies with IgA nephropathy. Transplant biopsies revealed the lowest possible values of C_T ratio (negative VCAM-1 amplification). VCAM-1 positive biopsies, however, such as from lupus nephritis, resulted in values even higher than those of the positive controls from MC culture. In one of the cases of mesangial proliferative lesions with unknown etiology and sufficient material for quantitative PCR, a positive amplification curve was also obtained.

Discussion

Our studies demonstrate for the first time the feasibility of quantitative PCR analysis of laser-microdissected glomerular slices from formalin-fixed, paraffin-embedded renal needle core biopsies using a specific gene of interest such as VCAM-1. Three glomerular slices of 5 μm thickness representing only femtograms of nucleic acids as starting material were sufficient to reliably obtain the predicted, quantitative PCR product. This result indicates a promising potential for gene expression analysis in future renal biopsies as well as in retrospective studies from renal pathology archives. The reproducibility of consecutive microdissection, mRNA extraction and reverse transcription as well as the high fidelity of quantification (as seen by replicate sample analyses, see Figure 2) using paraffinized or frozen material appears to be sufficient to rely on a positive result even from a single investigation. In addition, comparable levels of VCAM-1 could be detected analysing two biopsy cores simultaneously taken from the same patient with IgA nephropathy (Table 1). Furthermore, qualitative, semi-nested PCR analysis run in parallel with the quantitative PCR expectedly yielded an amplificate of identical size, providing valuable confirmation of the real-time PCR results. Finally, in numerous quantification runs, we avoided false positive results successfully by running appropriate no-template and no-amplification controls.

As the minimal amount of RNA obtained from a few glomerular slices cannot be quantified by a spectrophotometer, we used a ratio between the threshold of β-actin and VCAM-1 to calculate the expression level of the gene of interest, thus correcting for possible variations through the extraction procedure and enabling a more meaningful comparison between cases and different studies than absolute expression values would have done. Thus, we observed in our small biopsy series a clear distinction between cases positive or negative for increased VCAM-1 expression.

Importantly, positive VCAM-1 quantification could be revealed in several positive biopsy cases in which microscopically an inflammatory response was not readily visible, such as in IgA nephropathy and in mesangial proliferative lesions of unclear etiology. Surprisingly, all IgA cases studied showed a positive signal for VCAM-1. As cases of other entities analysed in the same quantitative PCR run resulted in positive as well as negative amplifications and all controls displayed correct negative results, this observation cannot easily be interpreted as false positive. As aggregated IgA may lead to a NF-κB-mediated stimulation of cultured human mesangial cells [14], our observation from human renal biopsies supports the idea that IgA nephropathy does not only represent a proliferative lesion but may well have an inflammatory component at least in some IgA cases. Currently, the role of VCAM-1 in IgA nephropathy remains speculative, and requires further investigation to evaluate whether this expression is of major functional importance or represents a non-specific bystander reaction.

In contrast to IgA nephropathy, VCAM-1 levels in one of the cases of lupus nephritis provided evidence for activation of the inflammatory pathway while another case did not, despite of their common histomorphologic classification (as diffuse proliferative). As activation of inflammatory signal transduction may contribute to the progression of a pre-existing...
mesangial proliferative lesion, these results clearly indicate the potential usefulness of quantitative, real-time PCR in future diagnostics and possible therapeutic implications.

Thus, in the near future, quantitative PCR analysis may enhance our understanding of pathophysiologic aspects of different renal diseases, once sufficient cases have been studied per disease entity and the range of expression levels for a specific gene have been defined.

However, there are also distinct methodological limits using formalin-fixed, paraffin-embedded renal biopsy cores. These are predominantly caused by the degree of degradation of RNA due to handling time before fixation, the use of a specific fixative and finally the paraffin embedding [15]. Four per cent of buffered formalin as fixative is essential, while a fixation duration of 2–6 h seems optimal. Unfortunately, the quality of formalin fixation as well as its duration cannot be determined from morphologic analysis alone. Whenever this information seems critical as well as for prospective cases, a close interaction with the respective nephrologist performing the biopsy is essential. The problem using archival paraffinized tissues is well known [16], and has been confirmed again for quantitative PCR by reports studying the influence of different fixatives on RNA quality [1]. Thus, a negative result for the quantification of the gene of interest can only be accepted as truly negative, if one has a positive control gene, indicating that the RNA is not completely degraded and if all positive and negative controls give the expected results. In this aspect, quantitative PCR is really not different as its ‘older sister’, qualitative PCR.

The success of our amplification is in part also due to avoiding known RNase destroyers and inhibitors of Taq polymerase such as diethyl pyrocarbonate [17]. Furthermore, and in contrast to many studies, our glomerular sections are representing very thin slices of non-solid tissue pieces more similar to the microdissection of single cells [18]. In currently published procedures for solid tissue blocks of 20 μm or more in thickness, full digestion with proteinase K is crucial before cell lysis [19]. The extraction protocols from both kits used in this study do not list this additional digestion step, while the composition of their lysis buffer as part of their patent remains a non-disclosed secret. Thus, the success of our extraction protocol may be in part due to properties of our special material such as delicate structures and a thickness of about one fourth of a cell, in part due to the special combination of ingredients in the lysis buffer itself. The lack of an additional digestion step is advantageous, since it allows us to reduce the necessary handling time by ~2 h.

Another important problem may arise from the limitations of the material available, particularly if thin needles are used to obtain the biopsy core. Most often, after sections for the routine analysis have been cut, there is not enough material, particularly glomeruli, left for evaluation. One has to postulate that a suitable biopsy should contain at least five, better 10 glomerular cross sections with completely intact Bowman capsule.

The decision to employ this novel technology is facilitated considering its rather limited time requirement. In our experience, one to three sections of 5 μm thickness can be cut and deparaffinized in ~1 h. The microdissection of a minimum of 10 glomeruli takes ~30 min each, while extraction and reverse transcription each need 1 h to complete. Thus, cDNA for analysis can be obtained within 1 working day. If a convenient overnight run is not possible, quantitative PCR for the transcript of interest and its control can be achieved the next morning simultaneously (provided the probes have different fluorescent labels) within 2–3 h depending on the number of cycles. Thus, compared with a routine overnight immunhistologic analysis, quantitative PCR is at least as fast.

For future diagnostic considerations, an additional, second (frozen) biopsy core could be advantageous, particularly regarding the improved results for RNA extraction from frozen vs formalin-fixed and paraffinized glomeruli. Besides an improvement in our daily immunhistologic analysis (i.e. particularly for IgG), its superior RNA yield could enable the study of many genes of interest, which is presently impossible by conventional immunhistology. In summary, quantitative PCR of microdissected glomeruli from formalin-fixed, paraffin-embedded needle core renal biopsies seems to be—within the limits set by RNA degradation—a useful and reliable method for evaluation of gene expression, being superior in its diagnostic value to any immunhistologic or simple morphologic evaluation. Its demonstrated potential to detect an activation of an inflammatory pathway as a possible progression factor of renal lesions could make it even a very promising method to obtain data useful for future therapeutic considerations.

Acknowledgements. We are indebted to Dr Holger Babbe for valuable discussion regarding the optimization of RNA extraction and Mrs A. Potratz, PE Biosystems, for her continued support with PCR quantification. Supported by the Deutsche Forschungsgemeinschaft (FR647/4) and the Imhoff-Stiftung, Cologne (both to J.W.U.F.).

Conflict of interest statement. None declared.

References


Received for publication: 8.9.02
Accepted in revised form: 5.12.02