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Gene Expression Analysis in Microdissected Renal Tissue

Current Challenges and Strategies

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Key Words

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Abstract

The architecture and compartmentalization of the kidney has stimulated the development of an array of microtechniques to study the functional differences between the distinct nephron segments. With the vast amounts of genomic sequence data now available, the groundwork has been laid for a comprehensive characterization of the molecular pathways defining the differences in nephron function. With the development of sensitive gene expression techniques the tools for a comprehensive molecular analysis of specific renal microenvironments have been provided: Quantitative RT-PCR technologies now allow the analysis of specific mRNAs from as little as single microdissected renal cells. A more global view of gene expression regulation is a logical development from the application of large scale profiling techniques. In this review, we will discuss the power and pitfalls of these approaches, including their potential for the functional characterization of nephron heterogeneity and diagnostic application in renal disease.

Introduction

Recent developments in molecular biology offer new opportunities for experimental and clinical medicine. With the completion of the draft sequence of the human genome the entire human genetic code is being deciphered [1, 2]. In addition, the genomes of laboratory animals such as mice and rats will be available in the near future. The integration of genomic information into biological function will be a major challenge for the biomedical community. A comprehensive analysis of the mRNA and protein expression in health and disease is considered to be an essential next step in this process. Current technical advances now allow a global analysis of the regulatory pathways active in a tissue or disease process [3].

The most promising immediate clinical application for patient care is the identification of mRNA expression patterns that help to characterize pathophysioloical phenomenon in diseased organs and their correlation with the diagnosis, prognosis and responsiveness to the different available treatments [4–6]. To date the most conclusive information available for diagnostic and therapeutic decisions in clinical nephrology is the analysis of the affected organ by fine needle biopsy. Biopsy material is analyzed primarily by histology [7]. The quantitative measurement of mRNA levels encoding functionally relevant molecules will add important information to this powerful diagnostic procedure in nephrology [8, 9].

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Diverse studies have evaluated gene expression in experimental kidney diseases, in renal cell culture studies and, to some degree, in human renal disease. These studies have identified – among others – a large number of cytokines, growth factors, chemokines and mediators of inflammation as contributing to renal failure. To simultaneously determine a wide range of mRNA molecules in microdissected tissues should further facilitate the characterization of nephron segment specific regulatory networks.

This review describes the methods currently available for nephron segment specific gene expression analysis, the initial results obtained with these techniques, and the major obstacles often encountered.

Obtaining Nephron Segments Suitable for Gene Expression Analysis

The analysis of nephron segment specific gene expression was simplified with the discovery of the polymerase chain reaction in the late 1980s. The nephrology community was quick to adapt this technique to the specific needs of the field [10–12]. Procedures were established to obtain renal tissue from experimental animals and regulatory networks were delineated along the nephron at the molecular level (see below and [13]).

Early on the translation of this approach to the analysis of human renal biopsy material was evaluated [14]. To obtain the necessary human renal biopsy tissue, technical and ethical considerations had to be taken into account. Renal biopsy is still an invasive procedure with a risk of gross hematuria of about 5–9%, need for blood transfusion of 0.1–3%, and nephrectomy of 0.02–0.05% [15]. Thus, any experimental protocol introduced must not interfere with the diagnostic evaluation of the renal biopsy. Therefore, sufficient tissue for clinical diagnosis has to be sampled, i.e. two biopsy cores of sufficient diameter and length [16]. Although former studies analyzed mRNA from an additional biopsy core [17, 18], new techniques have increased the sensitivity of the measurements allowing quantification of dozens of mRNA templates from just 10% of one biopsy core [19]. Examination of both cores under a stereo-microscope in the biopsy suite offers a reliable approach to determine whether sufficient tissue for diagnostic and research purposes has been sampled. In a current multicenter study separation of 10% of a biopsy core and mRNA-protected storage has not led to significant interference with routine diagnostics [19].

An alternative approach is to study gene expression on formaldehyde fixed, paraffin embedded tissues after completion of all necessary routine diagnostic evaluations. Combined with laser microdissection, this new methodology should enable retrospective mRNA analysis on nephron segments even after years of storage [20, 21]. As a further advantage this approach allows the selection of a defined histological structure for gene expression evaluation. Correlation of the mRNA expression profile with the clinical course adds additional power to this analysis. A major limitation of this technique is the low amount of mRNA that can be harvested from a fixed tissue specimen thus limiting the potential number of genes for analysis [20].

Addressing Nephron Segment Heterogeneity

The kidney consists of more than 20 different cell types and represents an organ of high functional complexity. As a consequence, a wide array of techniques, including micropuncture, microperfusion and microenzymatic analysis of nephron segments have been developed for experimental analysis [22]. The complex architecture of the kidney makes the dissection of nephron segments prior to gene expression analysis a crucial step in many analyses. In diseased kidneys mRNA expression is often differentially regulated in discreet nephron segments [23]. As a consequence, studies using whole kidney lysates often are confounded by a large variation in the expression levels obtained.

Two techniques have been developed for separation of nephron segments:

As an extension from microtechniques, the manual microdissection of murine glomeruli has allowed the analysis of glomerular-specific gene expression [10, 12, 13]. This technique has been successfully transferred to human renal tissue [14] and was employed in several studies [17, 24–27]. Microdissection and subsequent gene expression analysis of tubular nephron segments has also been reported [28–30]. The manual skill and time required for microdissection has limited the general application of this technique. A further problem with this approach is the loss of mRNA integrity during microdissection. This will be addressed in more detail below.

In recent years the use of laser microdissection has enabled the separation of nephron segments in frozen and fixed sections [20, 31–33]. Using renal tissue section areas of interest can be harvested for expression analysis via laser capture microdissection, manual harvesting with mi-

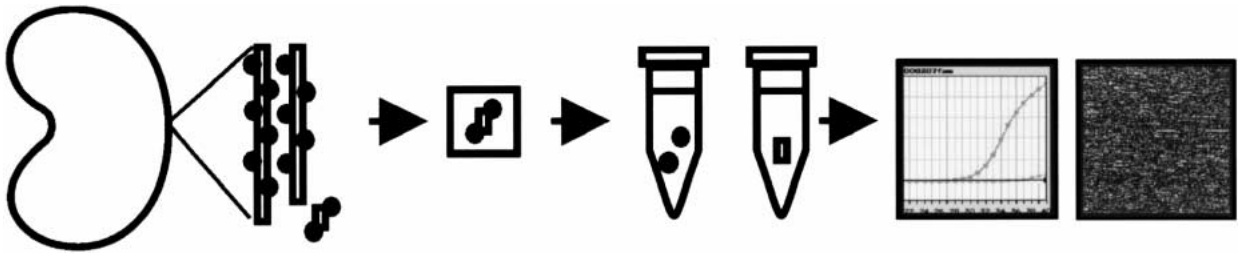

Fig. 1. Strategy for mRNA expression analysis on human biopsies in the multicenter setting. Immediately after renal biopsy, 10% of the two cores are separated and stored in RNase inhibitor. Under a stereo microscope glomeruli and tubulo-interstitium are separated by manual microdissection. The tissue can be stored for several years in RNase inhibitor. The gene expression analysis is performed by real-time RT-PCR or cDNA arrays.

cromanipulators or laser beam catapulting. These techniques require less manual dexterity and allow staining of the tissue for a clear delineation of the regions of interest. However, mRNA yield can be as much as two order of magnitudes lower as compared to manual microdissection of intact nephron segments [own unpubl. observation].

Maintaining Intact mRNA Templates

A central challenge in mRNA analysis is the inherent instability of the mRNA templates, particularly if material is to be obtained from clinical specimens. Like other tissues, the kidney is rich in RNases that induce a rapid degradation of mRNA. Several strategies have been employed to secure the template integrity for analysis:

Renal tissue can be immediately snap-frozen in liquid nitrogen and subsequently stored at –80°C. This has been effective in maintaining intact mRNA for months to years [34].

To preserve mRNA during the manual microdissection of nephron segments the RNase inhibitor vanadyl ribonucleoside complex was introduced by Moriyama et al. and served as the standard protocol for subsequent studies [10, 14]. With this approach the tissue still had to be microdissected directly after harvesting at 4°C, snap-frozen and stored at –80°C, significantly complicating the application in a clinical setting.

The new classes of RNase inhibitors such as RNAlater (Ambion) allows preservation of RNA even at room temperature for several hours [19]. This gives a convenient time window for the processing of the material. Using this approach renal tissue and RNA has been shown to remain intact at –20°C for more than three years [own unpubl. observation]. After storage the nephron segments can still be manually microdissected and the tissue remains suitable for immunohistochemical analysis [35]. This approach consistently allows high RNA preservation on microdissected renal biopsies (fig. 1) [19].

Optimizing RNA Yield

Using minimal tissue amounts as starting material, optimal RNA recovery is mandatory. In addition, all the cell types present in a nephron segment, such as a glomerulus, have to be lysed for RNA recovery. Initially, microdissected samples were permeabilized with Triton X [10] followed by direct reverse transcription of the obtained whole tissue lysate (in-situ RT). This protocol gave higher yields than an acid phenol based RNA extraction of the microdissected glomeruli [14]. Recently, a silica gel based RNA isolation was shown to be superior to in situ RT for tubular-interstitial and glomerular samples [19, 34]. RNA isolation also decreased the amount of contaminating genomic DNA and can, if required, be combined with removal of genomic DNA by DNase digestion.

Increasing Sensitivity of RNA Quantification

The low amount of available mRNA templates from small tissue compartments, such as microdissected nephron segments, requires the application of detection tech-

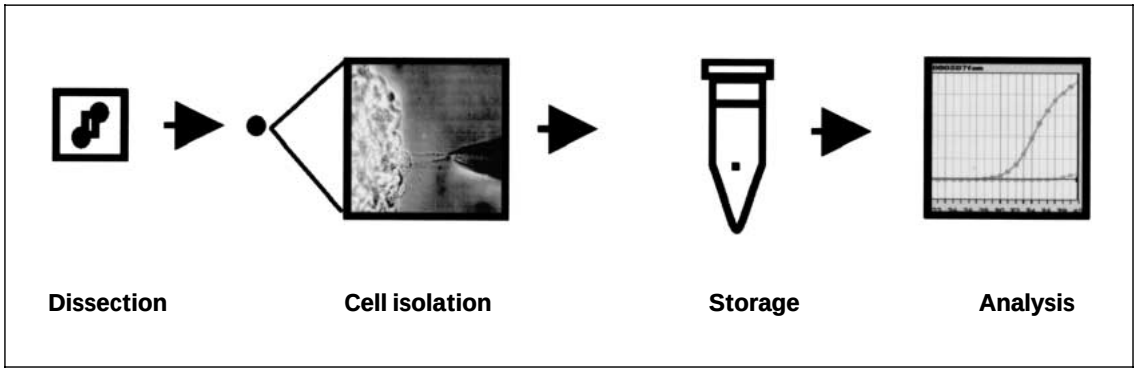

Fig. 2. mRNA quantification on microdissected single podocytes by real-time RT-PCR. After microdissection of a glomerulus a single podocyte can be harvested using a patch-clamp set-up (insert). The single cell is lysed by freeze-thaw steps and the RNA reverse transcribed. Quantification of cDNA is performed using real-time RT-PCR.

niques with extreme sensitivity. To this end, two approaches have been developed:

First, real time RT-PCR combines excellent sensitivity with quantification by the direct determination of amplicon numbers after each PCR cycle [36, 37]. Microtiter based assay formats allow for semi-automatization of the quantification procedure. This method has made it possible to scale down the assays up to the level of a single microdissected cell (fig. 2) [38, 39].

Oligonucleotide or cDNA arrays offer parallel quantitative mRNA investigation of hundreds or thousands of genes. A compelling alternative. However, the minimal amount of starting material is currently ~1 μg total RNA. Sufficient RNA is easily accessible from kidneys of animal models [40] or nephrectomies [41]. For biopsy studies, an entire human renal biopsy has be used for the sudy [42]. To obtain sufficient RNA from nephron segments a considerable amount of starting material has to be generated, i.e. by differential sieving techniques. An additional option would be to analyze microdissected nephron segments using cRNA that has been generated by RNA amplification prior to array analysis. Several protocols for the 'linear amplification' of cRNA have been developed for use with laser microdissected tissue other than kidney [43, 44]. The more than 1,000-fold amplification efficiency of these protocols can generate sufficient cRNA for the labeling reactions and array hybridizations. However, the critical issue of the linearity of the amplification, i.e. maintaining the different expression ratios between the diverse mRNA populations, is still a matter of conjecture.

Addressing the Focal Nature of Renal Disease

In renal disease, most processes affect the kidney in a focal manner and microdissected samples of a renal biopsy presenting only a small fraction of the kidney are prone to sample bias. In experimental studies, the sample error can be addressed by obtaining large numbers of the respective nephron segments from the same animal [23, 30]. In human biopsy studies, a comprehensive analysis of an adequate number of diseased kidneys in each diagnostic category is required.

Currently, two approaches appear to be feasible:

Prospective multicenter studies obtaining designated material for molecular studies may generate sufficient numbers of nephron segments in a central renal biopsy depository [45]. Depending on the study design (see above), adequate amounts of high-quality mRNA for screening approaches can also be gathered (fig. 1).

Access to mRNA obtained from formalin fixed paraffin embedded renal biopsy specimen will allow for retrospective studies on archival tissue (see above).

Outlook

A variety of animal models are currently being evaluated by nephron segment specific gene expression analysis. Comprehensive material and clinical data from patient populations are being generated for the study of human disease (fig. 1).

Messenger RNA expression screening experiments on human glomeruli have already identified novel regulatory

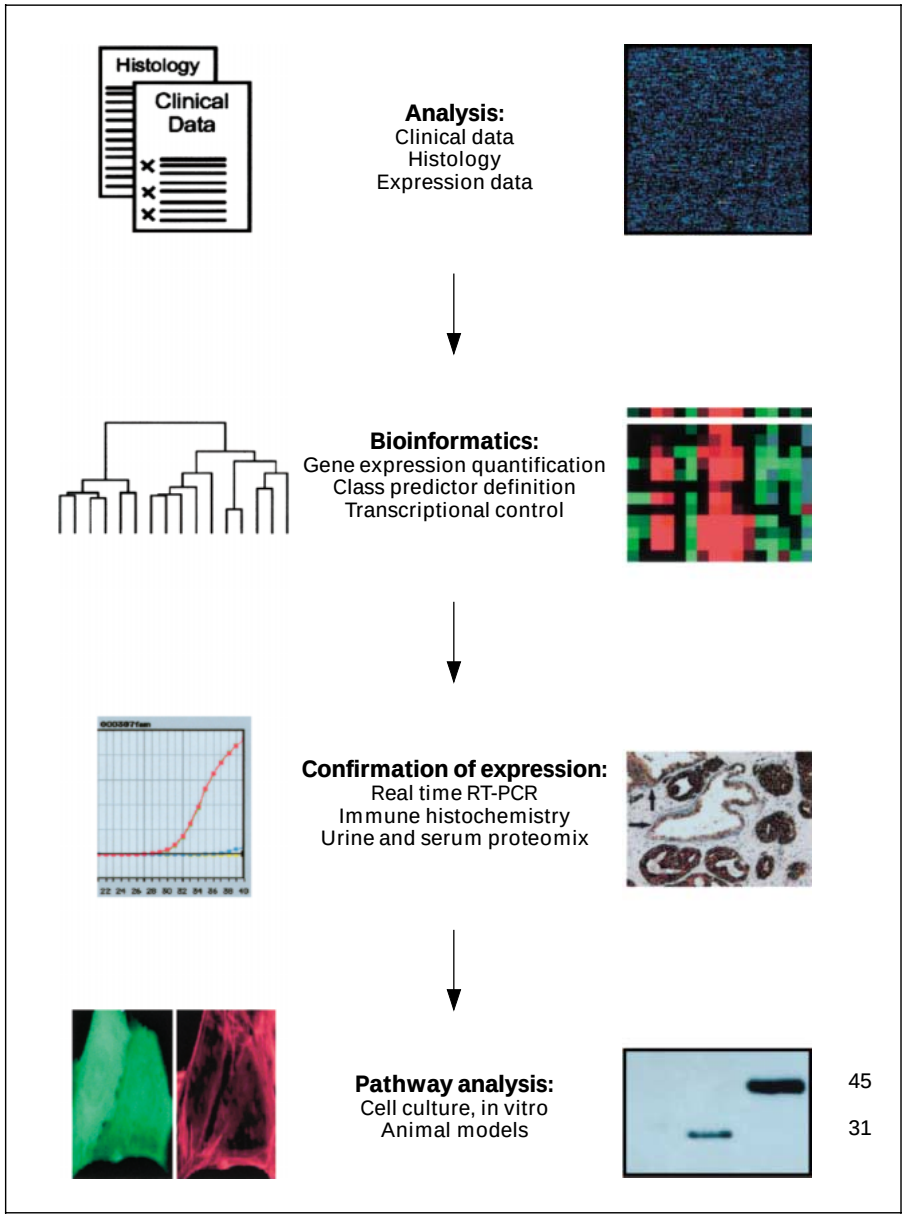

Fig. 3. Strategy for nephron segment specific molecular analysis. Gene expression of specific nephron segments is studied as given in the text. The expression profiles (right) can be analyzed in respect to the given experimental or clinical data and histology findings (left). Bioinformatic analysis aids in identifying disease-specific molecular pathways, i.e. by displaying complex expression pattern in graphical format (right) and by definition of similarities between samples (cluster dendrogram; left). Regulation of relevant expression profiles has to be confirmed with an independent approach, i.e. real-time RT-PCR (left) or immunohistology (right). Thereafter, the molecular pathways can be studied in detail using cell culture studies or animal models.

pathways active in renal disease [38, 46]. After evaluation in in vitro and in vivo systems (fig. 3), these molecules offer new diagnostic tools and also potential candidate genes for therapeutic intervention. As sufficient numbers of renal samples and clinical data become available, a comprehensive expression analysis of renal biopsies will allow a more global view of the regulatory mechanism activated in human renal disease. Data mining tools are being developed to display the complex expression patterns, to identify coregulated genes [47] and to dissect underlying transcriptional programs [48] (fig. 3).

The gene expression profiles may also be of use for molecular diagnostic information to supplement the modern pathological procedures [8, 49]. This approach has already been applied to the characterization of biopsy material obtained in oncology and hematology [4–6, 50, 51]. As gene expression profiles are a direct consequence of transcriptional programs, they have the potential to allow a more functional categorization of renal disease based on regulatory networks, rather than the descriptive classification used to date. If a stratification of diseases based on common molecular pathways can improve diag-

nostic impact regarding outcome and response to treatment remains to be determined.

Focussing the power of molecular analysis on specific nephron segments should provide a lot of answers, but certainly will give raise to many new questions. Exciting times ahead for the renal research community.

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