Endothelin peptides and receptors in human kidney

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1. Animal kidneys are exquisitely sensitive to the vasoconstrictor and antinatriuretic effects of the endogenous vascular peptide endothelin. Animal studies have implicated endothelin in cyclosporin A and ischaemia-mediated renal damage.

2. In man, endothelin levels are raised in various disorders. Orally active endothelin antagonists are now being developed, but little was known of endothelin's role as a renal peptide in humans. These studies therefore aimed to characterize endothelin peptides and receptors ETA and ETB in human kidney, to direct potential therapeutic endeavours.

3. Ligand binding, immunocytochemical, radioimmunnoassay and molecular biological studies were used to establish endothelin as a renal peptide in man.

4. The identification of species differences between man and rat directed further development of quantitative molecular biological methodology to permit analysis of endothelin receptors in human renal biopsies, and demonstrated perturbation of the system in the context of cyclosporin A therapy in renal transplantation.

INTRODUCTION

Once considered merely a lining layer of blood vessels with little active purpose beyond providing a barrier between blood and underlying tissues, the vascular endothelium is now known to be one of the body's most complex structures with many functions. The existence of an endogenous endothelium-derived vasoconstrictor was predicted from the early 1980s [1], but it was not until 1988 that Yanagisawa et al. [2] first reported the isolation and characterization of a 21-amino acid peptide, endothelin (ET), from the supernatant of cultured porcine aortic endothelial cells, whose prolonged and potent vascular effects have not been superseded by any other substance. Within a year, two further ET isoforms were described, designated ET-2 and ET-3 [3]. Their structure, which includes two intrachain disulphide bridges (Fig. 1), is unique among mammalian peptides, but the sarafotoxins (the active agents in the venom of the Israeli burrowing asp Atractaspis engaddensis) are highly homologous, and share ET's effects [4].

The endothelial linings of all contractile vessels secrete ET. Synthesis of mature (potentially functional) ET from the 212-amino acid precursor preproET involves two cleavage steps. First, the cleavage of two basic amino acid pairs (Lys51-Arg52 and Arg92-Arg93) produces the intermediate 38 amino acid peptide Big ET-1. The enzyme responsible remains to be defined, although furin has been suggested [5]. An unusual cleavage pattern between Trp21 and Val22 follows to yield the mature protein, and is catalysed by one phosphoramidon-sensitive endothelin-converting enzyme [6, 7].

The plasma half-life of exogenous ET is about 1 min [8] which probably reflects the wide distribution of its specific receptors, ETA and ETB. Slowed disappearance of exogenous 125I-ET-1 after bilateral nephrectomy in rats [9], together with the presence of ET in the urine, a significant amount of which appears to be excreted unchanged [10], provide evidence that the kidney excretes ET.

Previous animal (and more recently human) studies have shown the kidney to be very sensitive to exogenously administered ET-1, exhibiting arterial and arteriolar constriction more marked in the efferent circulation, together with falls in glomerular filtration rate, urine output and altered sodium balance (reviewed in [11]). Despite intense interest in the ET system, a definite pathophysiological role for this family of peptides has yet to be established. Indeed, much more has been made until recently of the search for ET's contribution to pathological events than of its potential place in the maintenance of vascular tone. In terms of the kidney, potential roles for ET in renally mediated haemodynamic control and electrolyte and fluid balance have been proposed, largely from animal studies, together with possible involvement in various pathological settings, namely ischaemic and contrast nephropathies.
and cyclosporin A (CyA) nephrotoxicity. The studies described here examined the ET system in the normal human kidney, and in the context of CyA therapy in renal transplantation.

LIGAND BINDING STUDIES: ET\(_B\) PREDOMINATES IN HUMAN KIDNEY

Ligand binding of \(^{125}\text{I}\)-ET-1 to 10\(\mu\)m-thick sections of fresh-frozen normal kidneys showed high, single-affinity specific binding to ET\(_A\) and ET\(_B\) receptors, with a \(K_D\) of 0.17\(\pm\)0.04 nmol/l [12]. Using the subtype-selective ligands BQ123 (an ET\(_A\) antagonist) and BQ3020 (an ET\(_B\) agonist, Fig. 1) in autoradiographic and binding studies it became evident that, unlike other tissues, human kidney is rich in ET\(_B\) in highest density on non-vascular structures. The ratio of ET\(_A\) to ET\(_B\) was 30:70%. About two-thirds of the specific binding was seen in the medulla, with increasing density towards papillae. The orally active non-peptide agent bosentan (Ro47-0203) was not selective between subtypes, and had a significantly lower binding affinity (\(K_D\) = 0.36\(\pm\)0.05 \(\mu\)mol/l) [13]. Similar \(B_{\text{max}}\) values, in the \(10^{-14}\) mol/mg protein range, were obtained with all three ligands. Macro- and microautoradiographic studies showed that ET\(_A\) localized to vessels, but unlike rat was not visualized within glomeruli. In contrast, ET\(_B\) was found on non-vascular structures such as tubules. Figure 2 shows an example of the binding of labelled BQ3020.

HUMAN KIDNEY AS A SOURCE OF ET

The presence of ET isoforms in normal samples of human kidney was sought by subjecting tissue
Endothelin in kidney

Fig. 3. Bright field photomicrographs (x 100) showing immunocytochemical localization of ET in the glomerulus (G) and endothelial lining of a blood vessel (BV) of normal human kidney. Panels A and B, specific staining; C and D, non-specific staining with preimmune sera; E and F, adjacent haematoxylin and eosin-stained sections.

extracts of cortex and medulla to reverse-phase HPLC separation followed by radioimmunoassay. These assays were only able to detect the presence of the ET-1 isoform and its oxidation product metsulphoxide-ET-1; total amounts of immunoreactive ET were 6.9±3.8 pmol/g wet weight in medulla and 4.5±1.6 pmol/g in cortex. The relative prevalence of peptide in medulla compared with cortex thus paralleled that of receptor [14].

Immunocytochemical staining using antibodies directed against the common C-termini of all three ET isoforms showed localization of immunoreactive ET to endothelia of vessels and of glomeruli. At high power this was seen to be cytoplasmic in all vessels down to a diameter of approximately 200 μm, but not in extra-glomerular capillaries. Faint tubular staining was observed at a 1:200 antibody dilution only (Fig. 3) [15].

The presence of mature peptide within kidney could arise as a result of renal clearance, so reverse transcriptase–polymerase chain reaction (RT–PCR) was used to demonstrate mRNA for ET [15]. Interestingly, RT–PCR using isoform-specific primer sets revealed mRNA for all three preproET isoforms. ET-2 mRNA was present in all samples of cortex and medulla analysed (n=7), and in two of five renal arteries and one of five renal veins. It has also been found in heart and endometrial tissue, but is undetectable in the circulation. ET-3 mRNA was also detected, in one vessel and five kidney samples. The significance of this apparent lack of translation remains unclear.

These studies confirmed human kidney as a source of ET. It was not possible to determine whether the metsulphoxide-ET-1 detected has a physiological role or is a function of early postoperative renal autolysis, but the rapidity of tissue preparation suggests that ET is oxidized endogenously.

DEVELOPMENT OF QUANTITATIVE RT-PCR ASSAY

Taken together, the results of these receptor binding and peptide studies provided good evidence for a significant interspecies difference in the renal ET system between man and rat. It was therefore imperative either to select a better animal model for study, or to extend as far as is possible the direct investigation of human tissue. The second alternative requires no extrapolations or assumptions to be made and in an ideal world would therefore be preferable, especially now that potentially therapeutic ET blocking agents are being developed. Appropriate characterization is needed which includes not only delineating the receptor subtypes accurately, but investigating ET’s pathophysiological role in man in detail. In terms of the kidney, this is particularly important because there may be serious consequences to the indiscriminate blocking of ET’s action if one receptor subtype actually has a beneficial role.

Various technical options included cell culture systems, although these introduce possible artefacts. Another logical step to take was to develop a means of analysing ex vivo human material obtained by biopsy. This entails study at the molecular level, since receptor binding studies are too unwieldy for such small specimens. Many highly significant observations have been made using Northern Blot analysis and the more sensitive RNase protection assay. However, needle-core biopsy tissue yields too little RNA to use these established methods; thus, to quantify ET receptor mRNA reliably, a novel quantitative fluorescent nested RT–PCR method, using a synthetic RNA standard, was developed and initially validated using normal human kidney.

The exponential amplification achieved by the PCR is extremely powerful; however, there are significant problems in making quantitative determinations. Firstly, the theoretical doubling of material with each cycle confers great sensitivity, but extrapolation back to the initial concentration is unrel-
able since small changes to or errors in the calculated rate of amplification will have a very large net effect in practice, and may be affected by tube-to-tube variations, making single-point analysis potentially unreliable. Quantitative determinations must be made while the reaction is still in the exponential phase, as in later cycles the reaction becomes 'saturated', mainly because of reannealing of DNA as its concentration rises, which prevents further oligonucleotide priming.

To overcome potential extrapolation errors an internal standard is required, against which specific mRNA can be compared, and either a dilution series is needed, or a range of different cycle numbers must be used. Various types of standard have been proposed, such as exogenous synthetic DNA or an endogenous cellular RNA. In brief, the former differs from authentic cellular material either by size or by presence or absence of a restriction site. Its main disadvantage is that it is DNA rather than RNA and must be added after the tissue RNA has been reverse-transcribed, which may vary in efficiency. An endogenous RNA standard is simpler, but if there are differences in optimal amplification conditions for the two sets of primers needed, then the amplification efficiencies may differ. If the amounts of mRNA are very different (as might occur if a housekeeping gene were used), the exponential phases might not coincide, making absolute comparison difficult. The standard mRNA might also itself vary with either pathology or experimental treatment.

A logical extension is to combine these two methods and use a synthetic cRNA which is added before the reverse transcription step. Standard and sample are treated identically, in the same tube throughout, using the same primer pairs, and the standard is not subject to biological variation. Additionally, the concentration of standard can be varied if necessary so that analysis can be performed in the exponential phase for amplification of both cDNAs and such that both are detectable. Finally, such a standard will be sufficiently similar in structure to the experimental cDNA to permit the assumption that in the critical first few cycles they behave similarly. This system, first described by Wang et al. in 1989 [16] is the most reliable method, provided that serial dilutions or variable cycle numbers are performed to construct standard and sample curves on every occasion, making day-to-day variations in reaction efficiency or conditions irrelevant. Figure 4 is a schematic representation of the assay.

To produce a cRNA standard, a plasmid was prepared into which double-stranded oligonucleotide sequences comprising 5' and 3' primer sequences were cloned in a directional manner between KpnI and HindIII sites. The standard thus contained a 580bp stuffer segment, providing an overall product size difference of about 300 bp. Two micrograms of tissue RNA were mixed in each assay with 600fg of standard. After nested PCR, one band of the appropriate size (ETA=299 bp, ETB=428 bp, construct ETA=628 bp, construct ETB=674 bp) was always obtained for each of sample and standard in each lane after electrophoresis.

telllite analysis technology is a sensitive method, which makes use of recently developed automated sequencing machinery and avoids the hazards of radioactivity. Figure 4 is a schematic representation of the assay.

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Seven normal cortices and eight medullas were assayed. The amounts of specific mRNA, in the amol/μg range, are depicted graphically in Fig. 5 and summarized in Table 1. The assay detected mRNA in the subattomolar range without difficulty. The amounts of specific mRNA found were similar to ranges quoted for various cytokine mRNA species [12]. The ETβ subtype accounted for about 70% of the receptor mRNA found, and the medulla contained the majority of the ET receptor mRNA, which correlates well with the previous ligand binding studies of expressed receptor protein. However, the temporal relationship between changes in mRNA and expressed protein levels is not known for this system. The tissues contained variable amounts of specific mRNA, although all within an order of ten. This is perhaps unsurprising in view of the heterogeneity of cell types in the kidney. The reproducibility of this assay is shown by interassay coefficients of variation of less than 5.5% [18].

**QUANTITATIVE PCR ANALYSIS OF TRANSPLANT BIOPSIES: POTENTIAL ROLE OF CYA**

This technique provided a tool with which to quantify changes in gene expression in the study of the human ET system. It is reliable, reproducible, sensitive and safe, and proved suitable for the analysis of an extremely small quantity of tissue, weighing only 3–10 mg. In subsequent studies the assay was used to investigate human diagnostic renal biopsies. However, it could equally well be used to study biopsy specimens from other organs, or cultured cells or explants.

Having established the relative abundance of ET receptor subtype mRNAs in normal human renal cortex and medulla, the next goal was to investigate quantitatively how ETα and ETβ receptor mRNA in the kidney might vary in renal dysfunction, in particular in the context of CyA therapy.

CyA is a lipid-soluble cyclic undecapeptide of fungal (*Tolypocladiurn inflatum*) origin which contains two unusual amino acids; sarcosine (not found in animals) and D-alanine. It exerts its potent immunosuppressive action by inhibition of CD4-positive T-cells [19]. Although CyA was developed principally for use as an immunosuppressive agent in solid organ transplantation, it is now emerging as an effective agent in conditions such as psoriasis, rheumatoid arthritis, early diabetes mellitus and asthma.

Several lines of evidence lend support to investigating the kidneys of patients on CyA. In clinical practice, cyclosporin’s two main troublesome adverse effects are hypertension (seen in the majority of its recipients) and nephrotoxicity, both of which are thought to be the result of vasoconstriction. Human CyA nephrotoxicity begins as a reversible decline in glomerular filtration rate [20] but can end in the development of interstitial fibrosis. The ability of renally derived human cells to synthesize ET [21, 22] and the recognition of raised plasma levels in CyA recipients [23] led, by analogy with animal experiments, to the hypothesis that CyA might mediate its renal vascular effects in man via the ET system. The prediction was made that in view of the vascular distribution of the ETα subtype, quantitative PCR analysis of ETα and ETβ mRNA would be more likely to reveal changes in the former. In the light of the observations made earlier regarding both interspecies and tissue distribution differences in ET receptor subtypes, these studies are also necessary in terms of the overall rationale of developing useful therapeutic agents to antagonize deleterious ET-mediated vasoconstriction.

Two groups of patients were compared: renal transplant recipients on CyA and those with native renal disease. Histologically normal kidneys (as above) provided a control group.

As displayed in Fig. 5 and Table 1, analysis of the three groups as a whole showed a highly significant decrease in the amounts of ETα mRNA present in transplant biopsies compared with either native biopsies or normal kidneys. Kruskal–Wallis testing yielded *P* = 0.003 compared with either normal kidneys or native biopsies. There was no difference between native and normal kidneys (*P* = 0.1). For ETβ there were no statistically significant differences. No significant correlations were found between mRNA levels and either age or creatinine level in any group.

While this was a fairly small sample population, highlighting the relative difficulty of obtaining human tissue, the results nonetheless achieved a high level of statistical significance. There are several possible interpretations of these data. Firstly, the downregulation seen could be a direct consequence of CyA therapy in these patients. The well-recognized phenomenon of CyA-associated hypertension and impairment of renal function in humans, with an increase of up to 25% in plasma

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**Fig. 5. Quantitative PCR analysis of cortices from normal kidney, native disease biopsies and transplant biopsies**
creatinine levels seen even in those whose CyA levels remained well within the therapeutic range, has so far defied functional explanation beyond the knowledge that vasoconstriction is central to these effects. If CyA directly stimulates ET to cause the vasoconstriction in the kidney, vascular ET<sub>A</sub> receptors would downregulate as part of a negative-feedback loop. By such a mechanism, only a small effect on ET<sub>B</sub> might be expected, since most of this subtype is non-vascular.

This explanation is also consistent with the elevated plasma ET levels in humans on CyA, and with Fogo et al.'s [24] protective pre-emptive administration of BQ123 in rats with acute CyA toxicity. Secondly, CyA might be responsible for direct endothelial toxicity such that ET is released from damaged cells, thereby producing unwanted vasoconstriction and a similar negative feedback via ET<sub>A</sub> on adjacent smooth muscle. However, none of the biopsies examined here showed this appearance, making this theory somewhat less tenable. Thirdly, CyA could exert its vasoconstrictive effects through an unrelated mechanism, the kidney's response being to attempt vasorelaxation via ET receptors. This is all the more relevant in the context of renal transplantation since neurogenic mechanisms have been removed. A fourth, less likely possibility concerns the underlying pathology in these tissues. Biopsies were performed in the transplant recipients because of suspected rejection. Several had histological features of early cellular rejection and one of humoral rejection, both of which affect the vessels. However the changes seen were mild in all cases, without medial disruption, and two were histologically normal. Since varying histological appearances accompany similar results, no trend can be established, but tissue damage itself does not account for these results.

It is of course possible that these results are simply a consequence of the transplantation procedure. However, the independent effect on vascular tone of this procedure has never been established. In order to tease apart all these possibilities, it would be necessary to study subgroups of transplant recipients who do and do not receive CyA, together with biopsies from those receiving CyA for other reasons, and larger groups in differing diagnostic categories. This raises practical issues; nowadays it is extremely unusual for CyA to be withheld, and the likelihood of numbers of patients in a non-transplant group requiring renal biopsies is remote. A more feasible study design would involve obtaining an initial, intraoperative biopsy and comparing the result with the diagnostic biopsies used here.

Although these results must be regarded as preliminary, the fact remains that cyclosporin causes deleterious vasoconstriction in man, and that human transplanted renal cortices display ET<sub>A</sub> but not ET<sub>B</sub> downregulation. Whether or not these two observations are ultimately directly linked or not, the general inference can be drawn that ET<sub>A</sub> antagonism might be therapeutically useful in this context.

The human renal salt and water handling effects of chronic CyA administration also warrant attention. Increased fractional sodium reabsorption and decreased proximal fluid delivery in CyA recipients [20] lends more weight to the idea of potential therapeutic benefit of ET antagonism as a protective vasodilatory mechanism in this context.

**CONCLUSIONS**

The studies presented here were carried out firstly to characterize ET and its receptors in the human kidney in vitro, and secondly, as important species differences were identified, to continue these human studies by investigating ET receptor mRNA quantitatively in transplant biopsies in the context of CyA therapy. These studies establish ET as a renal peptide in man. The downregulation of ET<sub>A</sub> shown by quantitative PCR analysis, together with differential interspecies ET receptor localization, suggest that in man, therapeutic attempts to block deleterious ET- or CyA-mediated effects should be via ET<sub>A</sub>-selective compounds.

In addition, the use of fluorescent quantitative RT-PCR has general applicability to the study of mRNA in human biopsy material, where the amounts of tissue are too small for older established techniques to be used. The technique could equally be applied to the temporal analysis of disease pathophysiology if repeated samples were taken and, if multiplexed, several different mRNA species could be studied simultaneously. The prerequisite of simultaneous amplification rates for sample and standard represents the crux of the assay and, should this be achieved, paves the way for the direct study of human disease processes.

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REFERENCES