Tissue expression and plasma levels of adrenomedullin in renal cancer patients

Jens MICHELSEN*, Helle THIESSON*, Steen WALTER†, Peter D. OTTOSEN‡, Ole SKØTT* and Boye L. JENSEN*

*Department of Physiology and Pharmacology, University of Southern Denmark, DK-5000 Odense, Denmark, †Department of Urology, Odense University Hospital, DK-5000 Odense, Denmark, and ‡Department of Pathology, Odense University Hospital, DK-5000 Odense, Denmark

ABSTRACT

The peptide AM (adrenomedullin) is stimulated by hypoxia through HIF-1 (hypoxia-inducible factor-1). The majority of human CC-RCCs (clear cell renal cell carcinomas) display mutations in the tumour suppressor protein von Hippel–Lindau, which leads to constitutively elevated HIF-1. We hypothesized that AM is increased in CC-RCC tumours and that AM is a plasma biomarker for CC-RCC. Tumours and non-malignant kidney tissue were obtained from patients that underwent unilateral nephrectomy. Blood samples were drawn at the day of surgery, 3–6 days after surgery and 4–5 weeks after surgery. AM mRNA and peptide expression in tissue and AM plasma concentration were determined. HIF-1α was localized in tissue by immunohistochemistry. AM mRNA was elevated in CC-RCC compared with adjacent renal cortex (6-fold, $n = 18$; $P < 0.02$). There was no difference in AM mRNA between cortex and non-CC-RCC tissue ($n = 7$). AM peptide concentration was elevated in CC-RCC tissue compared with adjacent cortex (4-fold, $n = 6$; $P < 0.02$), whereas there was no difference between cortex and non-CC-RCC tissue ($n = 5$). HIF-1α immunoreactivity was detected in the majority of cell nuclei in 76% of CC-RCC, consistent with constitutive stabilization. In non-CC-RCC, HIF-1α staining was focal. Before surgery there was no difference in plasma AM concentration between tumour types. Nephrectomy increased plasma AM significantly after 3–6 days and a similar pre-surgery level was observed after 4–5 weeks in both groups of tumour patients. We conclude that elevated tissue AM is a distinguishing feature of CC-RCC compared with other kidney tumours. Plasma AM is not suited as a tumour marker for this disease.

INTRODUCTION

The vasodilator peptide AM (adrenomedullin) was discovered in phaeochromocytoma cell extracts by its ability to initiate cAMP production in target cells [1]. AM is now considered as a paracrine factor associated with states of cellular growth and is expressed in various human malignancies [2]. AM is stimulated by hypoxia [3] under control of the basic helix–loop–helix transcription factor HIF (hypoxia-inducible factor)-1 [4]. HIF-1 is found in several types of common human cancers in a pattern compatible with reactive hypoxic induction, e.g. at the margins of necrotic areas in solid tumours or at the invading margins. However, in certain tumours, primary genetic alterations may be involved in the activation of HIF and its target genes rather than microenvironmental stimuli. Thus 50–75% of sporadic CC-RCCs [clear cell RCCs (renal cell carcinomas)], the most common RCC

Key words: adrenomedullin, hypoxia, kidney, renal cell carcinoma, tumour marker, vascular endothelial growth factor (VEGF).

Abbreviations: AM, adrenomedullin; AM-Gly, glycine-extended AM; CV, coefficient of variation; HIF, hypoxia-inducible factor; IRMA, immunoradiometric assay; mAM, mature AM; RCC, renal cell carcinoma; CC-RCC, clear cell renal cell carcinoma; RPA, ribonuclease protection assay; RT, reverse transcription; VEGF, vascular endothelial growth factor; VHL, von Hippel–Lindau; WHO, World Health Organization.

Correspondence: Dr Boye L. Jensen (email bljensen@health.sdu.dk).

type, display biallelic loss-of-function mutations in the tumour-suppressor gene *VHL* (von Hippel–Lindau) [5]. In the presence of oxygen, VHL is crucial for ubiquitin-mediated targeting of constitutively transcribed HIF-1α to proteasomal degradation [6]. Indeed, in the vast majority of CC-RCCs with *VHL* mutations, HIF-1α mRNA level is not changed, whereas HIF-1α protein is elevated significantly [7]. In these tumours, there is a close correlation between the level of HIF-1α protein and HIF-target gene products, e.g. VEGF (vascular endothelial growth factor) [7].

Based on these findings, we hypothesized that AM would be constitutively up-regulated in solid kidney tumours of clear cell type. The hypothesis was tested in human tumour tissue from nephrectomy samples. CC-RCC is an obvious target for better approaches for early detection of disease. AM is not stored in cells, but constitutively released at a rate that reflects transcription and translation. Since AM is expected to be elevated constitutively in all tumour cells in CC-RCC, we speculated that AM could be a potential serum marker for CC-RCC compared with other histological types of human renal and non-renal cancer. In the present study, we validated commercial immunoassays for the detection of AM in plasma and applied them to plasma samples from patients. Measurements were done in consecutive pre- and post-surgery samples of blood plasma from patients with CC-RCC and other renal neoplastic and non-neoplastic diseases.

MATERIALS AND METHODS

Subjects and design of study

The study was approved by the Institutional Review Board Ethical Committee (approval no. 20010035). All patients gave informed written consent for the use of tissue from the extirpated kidney and collection of blood samples. All samples in this study were obtained from randomly selected patients who underwent unilateral total nephrectomy for renal cancer at the Department of Urology, Odense University Hospital, Odense, Denmark. Blood samples were also obtained from six patients who underwent nephrectomy for reasons other than malignant disease. The pathological profiles of the patients who participated with consecutive blood samples are shown in Table 1. Before the blood sampling protocol began, several other patients donated nephrectomy specimens.

Collection of human blood and renal tissue samples

Antecubital venous blood was collected into cooled vacuum tubes containing EDTA and transferred to tubes with aprotinin. The samples were then centrifuged at 1500 *g* for 15 min at 4 °C, and separated plasma was stored at − 80 °C until assay. The first blood sample was obtained on the morning of surgery, the second was taken 3–6 days after surgery, and the third was collected 4–5 weeks after surgery. Immediately after removal, the kidney was transported to the Department of Pathology. A nephropathologist excised a portion of the tumour and normal tissue from the same kidney. Normal tissue was only collected from specimens where a segment of normal tissue, including both cortex and an intact papilla, was present. The normal tissue was dissected to give cortex and medulla. All tissue samples were frozen in liquid nitrogen and stored at − 80 °C. For histological analysis, a tissue block of the border zone between the tumour and non-malignant kidney was fixed in 4 % formaldehyde for 24 h and then embedded in paraffin. Tumours were diagnosed and classified according to the WHO (World Health Organization) classification [8].

Extraction of RNA and protein from tumour and normal kidney tissue

Total RNA was extracted from tumours and adjacent non-malignant renal cortex by using the RNeasy Midi Kit (Qiagen). Protein was isolated by homogenization of 200–250 mg of tissue in lysis buffer [1 mM Tris/HCl, 10 mM EDTA and 1 mM DTT with a protease inhibitor mix (Complete Protease Inhibitor; Roche Diagnostics) at pH 7.2]. Subsequently, 10 μl of Triton X-100 [10 % (w/v) final concentration] was added followed by centrifugation at 11 000 *g* at 4 °C for 10 min. The supernatant was removed and centrifuged again at 11 000 *g* at 4 °C for 10 min. Protein concentration was measured using a Bio-Rad Laboratories protein assay. Duplicate samples were diluted and compared with a BSA standard curve using spectrophotometry (Cary 50 Scan; Merck). Aliquots were stored at − 80 °C.

RT-PCR (reverse transcription–PCR) and cloning of PCR products

Human kidney RNA and CC-RCC RNA (1 μg) was reverse transcribed, and 50 ng of cDNA was used as a template for amplification by PCR as described previously [9]. The following DNA oligonucleotides (Invitrogen) were used for amplification: 5′-TCCGAAACCATGAACTTT-3′ (sense) and 5′-TCCACCAGGGTCTCGATT-3′ (antisense) for VEGF, covering bases 693–880 (188 bp; Genbank® accession no. NM_003376); and 5′-ACCGATTCACCATGGAGG-3′ (sense), and 5′-TCCAAATCACCAGCATCC-3′ (antisense) for HIF1-α, covering bases 254–500 (257 bp; Genbank® accession no. NM_001530). Primers were synthesized with restriction sites in the 5′ direction for BamHI (sense) and EcoRI (antisense). PCR products were subcloned in plasmid pSP73 (Promega) and sequenced as described previously [9]. A fragment of human AM cDNA (310 bp) has been cloned and sequenced previously [10].

Table 1 Characteristics of the patient cohort examined with consecutive collection of plasma before surgery, 3–6 days after surgery and 4–5 weeks after surgery

BP, systolic/diastolic blood pressure before surgery. The ratios shown under the Renography heading denote the estimated percentage contribution of right/left kidney to overall renal function. *Affected kidney that was excised. n.a., not available.

Patient number	Gender	Age (years)	BP (mmHg)	Pathological diagnosis (WHO criteria)	Renography (%)
1	Male	69	140/90	CC-RCC	57/43*
2	Female	79	125/80	CC-RCC	58/42*
3	Female	63	125/70	CC-RCC	48/52*
4	Male	60	144/75	CC-RCC	*0/100
5	Female	69	169/91	CC-RCC	*34/66
6	Male	63	175/95	CC-RCC	n.a.
7	Male	70	169/109	CC-RCC	48/52*
8	Male	71	130/60	CC-RCC	71/29*
9	Male	44	150/85	CC-RCC	n.a.
10	Female	49	120/80	Angiomyolipoma	*39/61
11	Male	74	140/87	Angiomyolipoma	*47/53
12	Male	37	135/75	Papillary-RCC	*49/51
13	Male	62	134/90	Papillary-RCC	*28/72
14	Male	73	167/103	Infiltrating urothelial carcinoma	*30/70
15	Male	41	130/85	Infiltrating urothelial carcinoma	50/50*
16	Male	68	147/80	Infiltrating urothelial carcinoma	*30/70
17	Female	77	195/100	Infiltrating urothelial carcinoma	87/13*
18	Male	81	180/70	Infiltrating urothelial carcinoma	*47/53
19	Female	56	170/90	Cystic nephroma	*36/64
20	Female	50	159/99	Nephroureterolithiasis	*6/94
21	Female	54	140/80	Nephrolithiasis (coral stone)	88/12*
22	Female	46	114/75	Nephrolithiasis (cyst)	*43/57
23	Male	35	125/95	Nephrolithiasis (coral stone)	*6/94
24	Male	52	110/60	Chronic inflammation	*10/90
25	Female	35	120/70	Kidney cyst	*2/98

RPA (ribonuclease protection assay) for AM, VEGF and HIF-1α mRNAs

The RPA was performed as described previously [9]. In addition to AM, VEGF mRNA was determined as a positive control for CC-RCC [7]. HIF-1α is stable at the mRNA level in malignant tissue and normal kidney cortex and it was used as a control for RNA quality and loading [7]. Initial experiments were done to assure surplus of probes and to elucidate the linear range of assays and feasibility of detecting all three mRNAs in one sample. RNA samples from 28 patients could be analysed simultaneously on one gel. RNA from matched samples of tumour and non-malignant renal cortex from one patient was always analysed on the same gel, each in duplicate. Thus four wells were occupied by RNA from one patient, and seven different patients were analysed in each assay. The duplicates consisted of 10 and 20 μg or, when possible, 20 and 40 μg of total RNA. By using this design it was possible to calculate the fold change of the relevant mRNA in the tumour compared with the adjacent cortex for each patient and thereby compare different assays. Protected probes were separated by urea/PAGE for 2 h and quantified as described previously [9].

Extraction of plasma

Because of the reported inaccuracy and poor validity of commercial assays for AM [11,12], we validated two commercial assays for detection of human AM in plasma: (i) an RIA involving a plasma extraction procedure (DRG Diagnostics), and (ii) an IRMA (immunoradiometric assay) with no plasma extraction (Shionogi) [13].

For the RIA, plasma was extracted using several extraction procedures: extraction 1, which is used routinely in our laboratory; extraction 2, as described by the manufacturer (DRG Diagnostics); and extractions 3a and 3b, which have been used by others previously to detect AM [11]. Plasma samples were prepared as described in Table 2 and applied on to conditioned Sep-Pak C_{18} cartridges. After washing the cartridges, samples were eluted using the solvents described in Table 2. Samples were collected in tubes containing Triton X-100 and evaporated to dryness under a stream of air in a 25 °C water bath overnight.

Immunoassays for AM

The RIA developed by DRG Diagnostics has a reported detection range of 1–128 pg/tube (approx.

Table 2 Extraction of plasma samples for use in the DRG Diagnostics RIA for AM

PATC buffer, 0.05 mol/l phosphate buffer (pH 7.4) with 1 g/l ATC (alkali treated casein), 1 ml/l Triton X-100, 0.1 g/l sodium EDTA and 0.2 g/l sodium azide. ATC was made by adding 5 g of casein to 20 ml of water, followed by 25 ml of 2 M NaOH and 100 μl of 10 M H_2O_2. The solution was left overnight at room temperature. After filtration (Whatman 541), the pH was adjusted with 1 M acetic acid to 7–8 and filtration was repeated using a Whatman GF/A filter. The pH was lowered to 4.5–4.6 with 1 M acetic acid. After 30 min, the supernatant was discarded, the precipitate washed with water, dissolved in aqueous ammonia and vacuum dried. The composition of PBSA buffer was as for PATC buffer, but with 5 g/l BSA (Sigma) instead of ATC. TFA, trifluoroacetic acid.

	Extraction protocols			
	Extraction 1	Extraction 2	Extraction 3a	Extraction 3b
Conditioning of Sep-Pak C_{18} cartridges	6 ml of 4% acetic acid in 96% methanol and 6 ml of 100 % methanol	6 ml of 4% acetic acid in 96% methanol and 6 ml of 100 % methanol	6 ml of 100% methanol	6 ml of 100% methanol
Equilibration	6 ml of water	6 ml of water	6 ml of water	6 ml of water
Sample preparation	1 ml of sample was added to 3 ml of 4% acetic acid with 0.1% TFA	1 ml of sample was added to 2 ml of 1% TFA	1 ml of sample was added to 1 ml of PATC buffer	1 ml of sample was added to 1 ml of PBSA buffer
Washing	6 ml of Mili-Q water	2 ml of 1% TFA	6 ml of saline (9 g/l NaCl)	6 ml of saline (9 g/l NaCl)
Elution	3 ml of 4% acetic acid in 80% methanol with 0.1% TFA	2 ml of 60% acetonitrile in 1% TFA	3 ml of 80% isopropanol with 0.013 ml/l HCl	3 ml of 80% isopropanol with 0.013 ml/l HCl
Collection	10 μl of 0.1% Triton X-100	10 μl of 0.1% Triton X-100	10 μl of 1% Triton X-100	10 μl of 1% Triton X-100

0.17–21 fmol/tube; molecular mass of AM is 6029 g/mol) and uses a polyclonal antibody which does not discriminate between active mAM (mature AM) and the intermediate-inactive form AM-Gly (glycine-extended AM) [13]. The IRMA developed by Shionogi measures total AM (mAM + AM-Gly) and uses two monoclonal antibodies against different regions of human AM. The assay has a detection range of 2–500 fmol/ml and does not require extraction of plasma. All procedures were conducted according to the manufacturer's instructions. Final samples in both kits were counted in a γ-counter (Cobra; Packard).

Assay characteristics

Plasma extraction protocols were evaluated by measuring recovery and precision by within-assay and between-assay CVs (coefficients of variation). Accuracy was determined by measuring linearity of serially diluted plasma samples. Recovery was determined by adding 45.5 fmol/ml (Shionogi) or 49.8 fmol/ml (Phoenix Pharmaceuticals) exogenous AM to a plasma sample from a healthy human volunteer. Recovery of radioactive label was measured by applying 10200 c.p.m. of ^{125}I-labelled AM to the Sep-Pak C_{18} cartridges.

Immunohistochemistry

Tumour tissue was fixed in 4% formaldehyde at 4°C for at least 24 h, dehydrated and embedded in paraffin by standard procedures. Sections (4 μm thick) were mounted on glass slides. All sections were stained under the same conditions with a specific anti-AM antibody (H-010-01; Phoenix Pharmaceuticals) as described previously [10]. Staining for HIF-1α was done basically as described previously [7] using an anti-(human HIF-1α) antibody (NB 100-123; Novus Biologicals) at a dilution of 1:1000. Antigen retrieval was done by pressure-cooking for 10 min with target retrieval solution (S1599; DAKO) and the Catalyzed Signal Amplification System (DAKO). Light counterstain with haematoxylin was used. Negative controls were performed using 5% (w/v) dry milk and non-immune mouse IgG for amplification. Two investigators independently evaluated the labelled sections in a blinded fashion. We used the method described by Zhong et al. [14] to score the tumour sections as follows: −, no staining, + nuclear staining in less than 1% of cells, ++, nuclear staining in 1–10% of cells, +++ nuclear staining in 11–50% of cells and ++++, nuclear staining in >50% of cells. When scoring of a sample differed, the section was re-evaluated by both reviewers simultaneously. Sections were inspected in a BX51 Olympus microscope.

Statistics and data presentation

Data obtained for mRNA expression and peptide concentration in paired samples of tumour and normal cortex were evaluated using one-sample Student's t test on the average tumour/cortex ratio. The null hypothesis was that there was no difference between cortex and tumour in which case the hypothetical ratio would be 1. Data obtained by consecutive plasma sampling in the same patient were evaluated by Student's t test for paired observations. When two independent groups were compared (e.g. healthy controls and patients with malignant kidney disease) Student's t test was used to evaluate the level of statistical significance. $P < 0.05$ was considered significant. Values are expressed as means ± S.E.M.

RESULTS

Patient characteristics

A total of 25 patients were examined by three consecutive blood samples before and after nephrectomy, 19 of whom had malignant kidney disease (Table 1). Plasma creatinine was within the normal range before surgery (98.5 ± 3.5 μmol/l) and increased significantly 2–3 days after nephrectomy when data from all patients were pooled (114.5 ± 4.8 μmol/l, $n = 20$; $P < 0.02$, as determined by a paired Student t test). Renography was done in all patients before surgery and showed that, in patients with malignant renal disease, the kidneys did not display similar function (Table 1; $n = 18$; $P < 0.01$, as determined by a paired Student t test). The affected kidney in each case had the lower performance. In six patients who underwent nephrectomy for non-malignant indications, this difference was more pronounced (Table 1; $P < 0.001$, as determined by a paired Student t test). There was no significant difference in blood pressure between patients with CC-RCC and non-CC-RCC (mean systolic pressure, 153 ± 7 compared with 152 ± 8 mmHg; mean diastolic pressure, 90 ± 8 compared with 85 ± 4 mmHg), but patients with neoplastic disease exhibited significant ($P < 0.02$) systolic hypertension compared with the patients without neoplastic renal disease (mean systolic pressure, 152 ± 5 compared with 128 ± 8 mmHg; mean diastolic pressure, 87 ± 3 compared with 80 ± 6 mmHg).

AM, HIF-1α and VEGF mRNA expression in homogenates of human renal tumours and adjacent cortex tissue

Cloned cDNAs matched the published sequences by between 96 and 100%. There was a linear relationship between the quantity of total RNA assayed (5–40 μg) and protected radioactivity in all three hybridization products, both when one probe was applied in one RNA sample and when all three probes were added simultaneously in one sample (Figure 1). This indicates that the probe was present in excess. Hybridization products displayed the expected molecular size and, in the absence of template RNA, probes were completely digested whereas, in the absence of RNAse, probes remained intact (Figure 1). In CC-RCC patients, average AM mRNA ratio between the tumour and adjacent non-malignant renal cortex was significantly higher than 1 ($P < 0.02$, as determined by a paired Student t test) (Figure 2). Of note, in four of the 18 CC-RCCs analysed, the AM ratio was at or below 1.5, indicating no significant difference. In non-CC-RCC, there was no difference in AM mRNA level when the tumour tissue was compared with adjacent renal cortex (Figure 2). VEGF mRNA level was also increased significantly in CC-RCC compared with adjacent non-malignant renal cortex ($P < 0.001$, as determined by a paired Student t test), but was not significantly different in non-CC-RCC tissue compared with adjacent renal cortex (Figure 2). As shown previously [7], HIF-1α mRNA level was similar in all samples investigated, both malignant and adjacent cortex tissues, which indicated similar quality and loading of RNA.

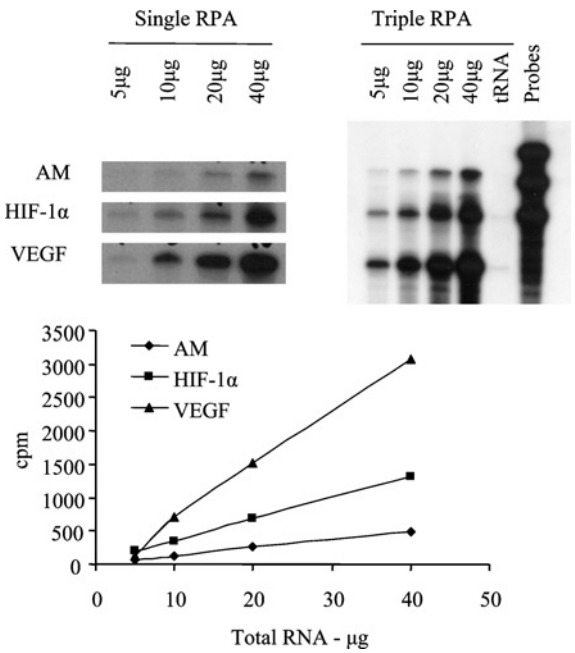

Figure 1 Validation of RPAs for AM, VEGF and HIF-1α mRNAs

Left-hand panel, the result of three separate RPAs (single RPA) in which increasing amounts of total RNA were hybridized with AM-, VEGF- and HIF-specific antisense RNA probes. Right-hand panel, the result of a similar RPA where all three specific probes hybridized simultaneously in each RNA sample (triple RPA). The penultimate lane shows the hybridization with 40 μg of yeast tRNA (negative control). The last lane shows cRNA probes. Quantitative evaluation of the gels is shown below.

Validation of AM peptide assays

The RIA kit from DRG Diagnostics was applied first and different extraction procedures were tested (see Table 2) in an attempt to optimize the results. No meaningful results were obtained from extraction 2 (results not shown), and extractions 1 and 3 exhibited low recoveries of added AM, displaying high between-assay and intra-assay variation and non-linear dilution curves with low accuracy (Table 3). Thus, despite continuous dilution, AM was detected at similar concentrations. Recovery of radiolabelled AM was comparably better, which indicated that the extraction procedure was effective, but that downstream assay detection was not quantitative. The assay was therefore not used subsequently. The IRMA kit (Shionogi) did not require extraction and exhibited far better performance (Table 3). Assay accuracy was demonstrated by the linearity of AM concentrations measured in serially diluted plasma samples (Table 3). The assay exhibited low within-assay and between-assay CVs that corresponded to values given by the manufacturer, both with control plasma (within assay, $n = 5$; and

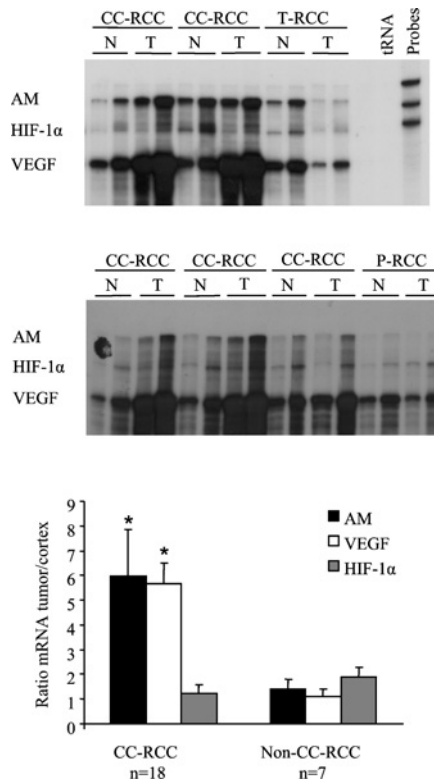

Figure 2 Expression of AM, VEGF and HIF-1α mRNAs in CC-RCC and non-CC-RCC and adjacent non-malignant cortex tissue

Top and middle panels, results of two separate RPAs where malignant tissue and non-malignant cortex from individual patients were analysed in duplicate (10 and 20 μg or 20 and 40 μg total RNA samples). T-RCC, transitional RCC; P-RCC, papillary RCC; N: non-malignant cortex, T, tumour. Lower panel, ratios between AM, VEGF, and HIF-1 mRNA in tumours and in adjacent non-malignant renal cortex measured by RPA. There was a significantly higher level of AM ($^*P < 0.02$) and VEGF ($^*P < 0.001$) mRNAs in CC-RCC compared with cortex, but not in non-CC-RCC. Values are means ± S.E.M of individual patient ratios between tumour and non-malignant tissue.

between assays, $n = 8$) and with plasma spiked with 45.5 fmol/ml of exogenous AM (within assay, $n = 5$; between assays, $n = 9$; Table 3). Recovery was excellent with AM peptide obtained from Shionogi, but not from a different manufacturer (Table 3).

Assay validity for tissue homogenates was tested. Post-homogenization recovery was between 33.7 and 46.7 %, but pre-homogenization recovery was only found to be between 5.7 and 7.9 %. Between- and within-assay CVs were not measured for the tissue homogenates because of limited tissue availability.

AM peptide content in homogenates of renal tumour tissue and adjacent renal cortex

AM peptide concentration was determined by IRMA in tissue homogenates and normalized for protein concentration. There was no difference in AM content between normal adjacent cortices from kidneys with CC-RCC and non-CC-RCC and, therefore, the values were pooled. There was a significantly higher ($P < 0.02$, as determined by an unpaired Student t test) AM concentration in homogenates from CC-RCC (1.82 ± 0.3 fmol/mg of protein; $n = 13$) compared with non-malignant renal cortex (0.41 ± 0.06 fmol/mg of protein; $n = 17$). There was no significant difference in AM concentration between non-malignant cortex (0.41 ± 0.06 fmol/mg of protein; $n = 17$) and non-CC-RCC (0.55 ± 0.09 fmol/mg of protein; $n = 6$) homogenates. In a number of cortex samples, the AM concentration was below the detection limit of the assay while AM in the tumour was detectable. In those paired tissue homogenates where AM could be detected both in cortex and tumour, the tumour/cortex AM concentration ratio was calculated. The AM ratio was significantly higher ($P < 0.006$, as determined by an unpaired Student t test) than 1 in CC-RCC (5.50 ± 1.07; $n = 9$) but not in non-CC-RCC (1.67 ± 0.28; $n = 5$).

Pre- and post-nephrectomy plasma concentration of AM in patients with renal tumours

Average plasma concentration of total AM (AM-Gly + mAM) in eight healthy human control individuals (four male and four female; median age, 37 years) was 8.14 ± 0.72 (range, 5.37–11.20) fmol/ml. Before nephrectomy, there was no difference between total plasma AM concentration in patients with CC-RCC (median age, 69 years; $n = 9$) compared with patients with a renal tumour of a different histological classification (median age, 65 years; $n = 10$) (Figure 3). Pre-surgery plasma AM concentration in all patients with malignant renal tumours was 12.8 ± 0.9 fmol/ml ($n = 20$), which was significantly higher ($P \leqslant 0.004$, as determined by an unpaired Student t test) than in healthy control individuals. At 3–6 days after surgery, there was a significant increase in plasma AM level in both groups of tumour patients ($P < 0.05$ and $P < 0.005$ in CC-RRC and non-CC-RCC respectively, as determined by paired Student t tests; Figure 3). After 4–5 weeks, plasma AM concentration was not statistically different from the pre-surgery level in the nephrectomized patients (Figure 3).

We measured plasma AM in six patients who underwent unilateral nephrectomy for indications other than malignant kidney disease (median age, 48 years; Table 1). The pre-surgery AM concentration was 15.6 ± 3.1 fmol/ml, which was significantly higher ($P \leqslant 0.02$, as determined by an unpaired Student t test) than in healthy controls. At 3–6 days after surgery, plasma AM was 24.1 ± 3.9 fmol/ml and, after 4–5 weeks, the level was 15.9 ± 1.9 fmol/ml. These values were not significantly different from pre-surgery plasma AM levels (as determined by a paired Student t test).

Table 3 Comparison of commercial immunoassays for AM without and with extraction procedures

Numbers of repeated measurements are indicated in the text. Recovery was determined with exogenous AM peptide purchased from either Phoenix Laboratories or Shionogi. Hot recovery, recovery of radiolabelled AM. n.d., Not determined.

		DRG Diagnostics kit		
	Shionogi kit	Extraction I	Extraction 3a	Extraction 3b
Recovery of AM (%)				
AM (Phoenix Laboratories)	35.1	6.4	32	31.7
AM (Shionogi)	100.7	n.d.	n.d.	n.d.
Curve fit (r value)	1	0.89	0.93	0.92
Within-assay CV (%)				
Pooled sample	4.3	25	16.7	21.7
Pooled sample spiked with AM	3.2	18.2	6.8	11
Between-assay CV (%)				
Pooled sample	8.3	35.9	n.d.	n.d.
Pooled sample spiked with AM	9.8	n.d.	n.d.	n.d.
Hot recovery (%)	n.d.	84.1	83.5	79.1

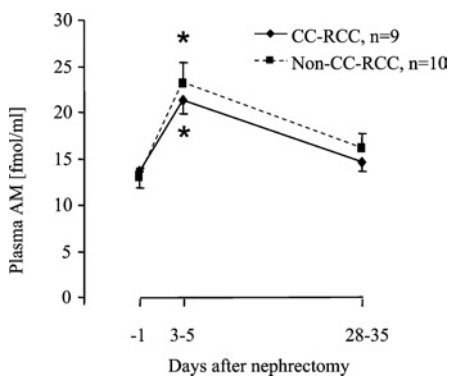

Figure 3 Total AM (AM-Gly + mAM) concentration in plasma from patients with malignant kidney disease before and after nephrectomy

Values are means ± S.E.M. There was no significant difference between plasma AM levels in patients with CC-RCC compared with patients with non-CC-RCC. Plasma AM increased significantly in both groups of patients 3–5 days after surgery ($^*P < 0.05$ for CC-RCC; $^*P < 0.005$ for non-CC-RCC). At 4–5 weeks after nephrectomy, plasma AM had returned to pre-surgery levels.

Immunohistochemical labelling of renal tumour tissue for AM and HIF-1α

A series of renal tumour samples were labelled for AM by immunohistochemistry (CC-RCC, $n = 25$, papillary RCC, $n = 1$; urothelial carcinoma, $n = 3$; angiomyolipomas, $n = 2$; oncocytoma $n = 2$; Figure 4). No obvious differences in staining intensity were found between the different tumour types. Thus AM was detected in the cytoplasm of tumour cells in the vast majority of CC-RCC samples, whereas little if any staining was associated with the stroma (Figure 4A). Most non-CC-RCC samples also displayed a variable degree of AM-positive tumour cells. Of note, AM-labelled tumour cells were homogeneously distributed in CC-RCC tissue (Figure 4A and 4B). In the adjacent non-malignant renal tissue, AM was associated with distal tubules (Figure 4C) and collecting ducts regardless of the type of malignancy. In the absence of the primary anti-AM antibody, no labelling was detected (Figure 4D).

Randomly selected CC-RCCs ($n = 17$) and non-CC-RCCs ($n = 12$) were stained for HIF-1α in order to ascertain the correct diagnosis of CC-RCCs and to compare the pattern of distribution with non-CC-RCCs. Normal kidney tissue displayed very little staining for HIF-1α and, in several samples, no signals were detectable (Figure 5D). In some cases HIF-1α-immunoreactive protein was observed in normal tissue adjacent to the invading tumour (Figure 5F). In complete contrast with normal kidney tissue, 13 out of 17 (76%) of CC-RCCs exhibited homogeneous strong labelling (+++ and ++++) that was associated with tumour cell nuclei (Figures 5A and 5B). In CC-RCC, localization of HIF-1α-positive cells did not depend on position within the tumour, e.g. at margins, in cysts or close to vessels (Figures 5A and 5B). Incubation with non-specific mouse IgG or omission of primary anti-HIF antibody did not result in labelling of CC-RCC, despite the use of signal amplification (Figure 5C). Immunostaining for HIF-1α in tumours diagnosed as non-CC-RCC was heterogenous and sparse (Figures 5E–G). HIF-1α labelling was associated with cell nuclei that typically accumulated at sites associated with necrosis or inflammatory cell infiltrates (Figure 5E) or at the invading margin (Figure 5F; transitional RCC). In renal papillary tumours, HIF labelling was typically associated with single cells in the wall of intratumoral cysts, whereas the bulk of tumour cells were negative (Figure 5G). Non-CC-RCCs received low scores (all were + and ++), indicating that the estimated number of HIF-positive nuclei was < 10% in all 12 samples investigated.

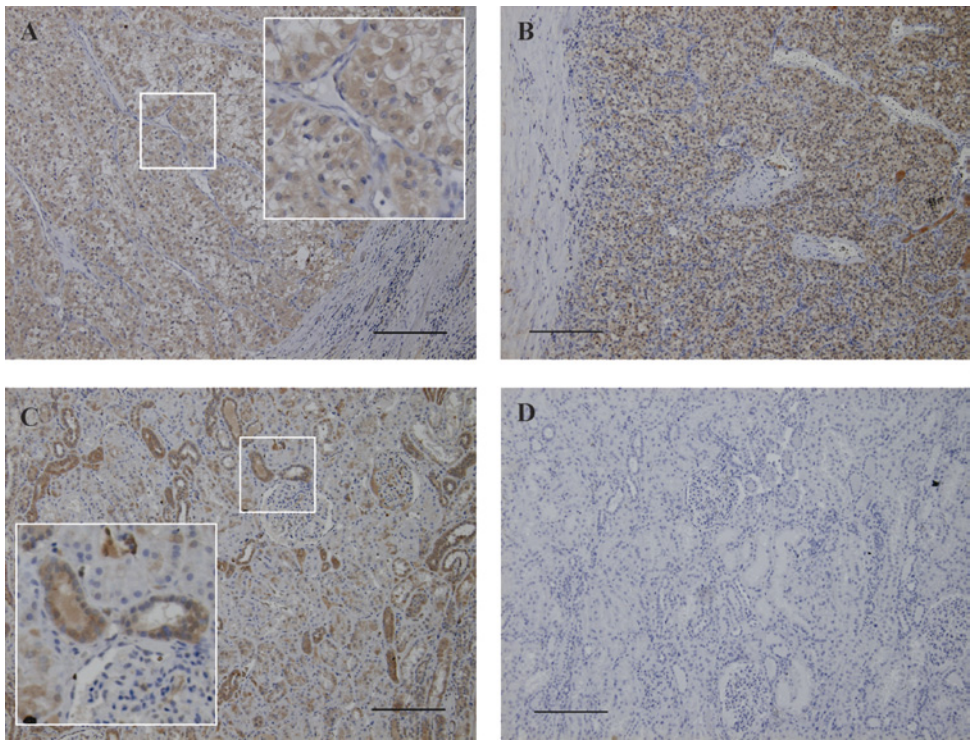

Figure 4 Immunohistochemical analysis of AM expression in CC-RCC

(A and B) Immunohistochemical labelling of CC-RCC for AM. The invading margin is seen. AM labelling is homogeneously associated with tumour cells. Inset in (A), a larger magnification of typical tumour stroma, which is negative for AM, and AM-positive clear cells within the tumour. (C) In non-malignant adjacent kidney cortex, AM labelling was associated with distinct tubules. Inset, labelling of tubules close to glomeruli, most probably distal convoluted tubules. (D) Negative control, where the primary antibody was omitted. Scale bars, 200 μm.

DISCUSSION

In the present study we tested the hypotheses that the vasodilator peptide AM is constitutively elevated in CC-RCC tissue and that AM is a plasma marker for CC-RCC. The data have shown that AM mRNA and protein were significantly and selectively elevated in CC-RCCs compared with adjacent renal cortical tissue. The majority (76 %) of CC-RCCs displayed strong homogeneous nuclear labelling for HIF-1α, whereas none of the examined non-CC-RCCs had this pattern of HIF-1α distribution. Plasma AM concentration was significantly increased in patients with renal malignant disease compared with a group of healthy controls. Plasma AM in patients with CC-RCC was not different from that in patients with other renal malignant tumours. Rather than the decrease in plasma AM predicted by our hypothesis, we observed that nephrectomy led to a significant increase in plasma AM concentration in all patient groups 3–6 days after surgery. Plasma AM subsequently stabilized to pre-surgery levels 4–5 weeks after nephrectomy.

The present findings and those published previously [11,12] of unreliable detection of AM in plasma by immunoassays indicate that commercial assays should be subjected to rigorous validation before general use in clinical settings. Assay accuracy is vital because AM is known as a 'sticky' peptide and most reported changes in plasma AM are of a small magnitude. We found that, in our hands, the IRMA kit for total AM performed far better than the RIA and was valid and reproducible on all criteria tested. We are not aware of previous studies on plasma AM concentrations in renal cancer patients. Plasma AM is elevated in patients with lung cancer and gastrointestinal cancers [15], whereas it is not different from controls in patients with breast cancer [16]. Because localized hypoxia is an inherent property of most solid tumours, it is not surprising that plasma AM is elevated in many common cancer types. We observed that plasma AM was also elevated in six patients admitted for nephrectomy but without malignant disease. A common denominator for the patients that underwent nephrectomy was impaired function of the affected kidney, as indicated by renography. Renal impairment is associated with increased plasma AM concentration [17], but judging from plasma creatinine concentration overall renal function was not affected differently in the patients. At 4–5 weeks after nephrectomy, plasma AM was still significantly higher than in healthy controls. This indicates that the elevated AM level is not related to the diseased kidney. Systolic blood pressure was significantly higher

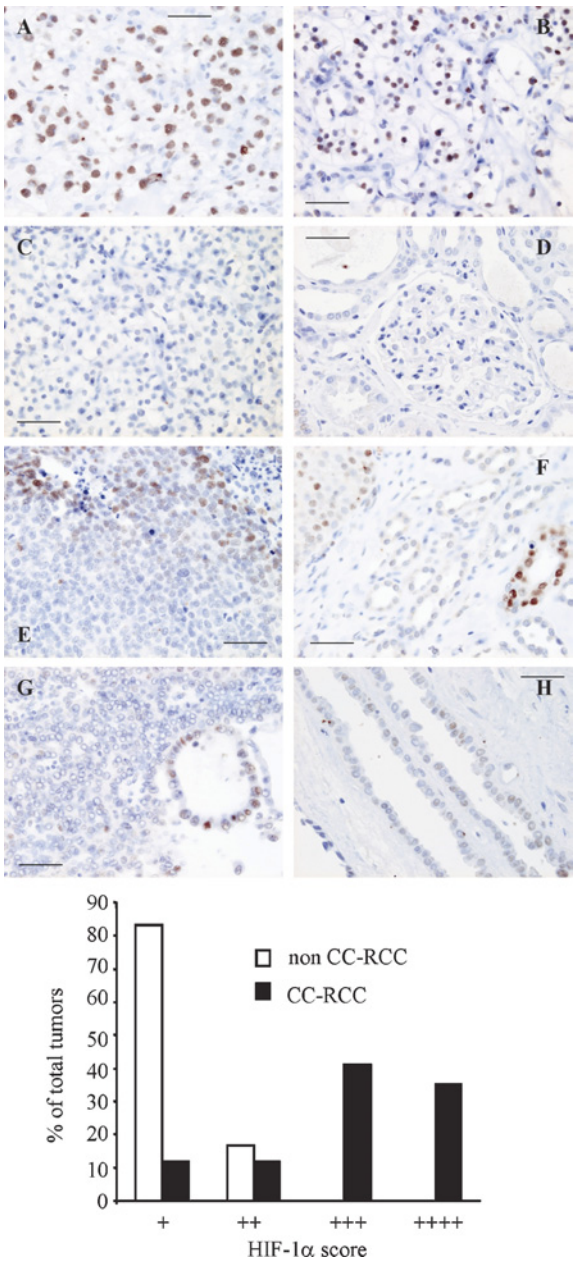

Figure 5 Immunohistochemical analysis of HIF-1α expression in kidney tumours

Analysis of sections from typical CC-RCC (A and B), negative control for CC-RCC in which non-immune mouse IgG was used (C), normal kidney cortex (D), transitional RCC (E and F), where (F) shows the invading margin (upper left) and a HIF-1α-positive collecting duct in adjacent renal medulla, papillary RCC with few HIF-1α-positive cells in the wall of a cystic structure (G), and normal kidney medulla (H), showing two collecting ducts and the papillary surface epithelium (lower-left corner). Scale bars represent 50 μm in all micrographs. Lower panel, comparison of the distribution of HIF-1α immunohistochemical labelling in non-CC-RCCs and CC-RCCs. Tumours were scored in a blinded fashion by two independent observers as follows: $-$, no staining; $+$, nuclear staining in less than 1 % of cells; $++$, nuclear staining in 1–10 % of cells; $+++$, nuclear staining in 11–50 % of cells; and $++++$, nuclear staining in $>$50 % of cells. Values represent the distribution of scores as a percentage of the total number of tumours analysed.

in patients with renal cancer compared with patients admitted for nephrectomy without neoplastic disease, but AM was elevated equally in both groups. It is not clear what the mechanism is that underlies elevated plasma AM in the group of nephrectomized patients.

Most sporadic CC-RCCs (50–70 %) exhibit mutations in the tumour suppressor gene *VHL* [5,7]. We did not perform mutational analysis for *VHL* in the present study, but relied on classical pathological criteria. The tissue and plasma measurements of AM showed very little variation. Thus it is unlikely that a subgroup of patients diagnosed as 'clear cell' but without *VHL* mutations display markedly different plasma AM concentrations. In support of correct pathological diagnosis, the majority of clear cell tumours in the present study displayed uniform nuclear labelling for HIF-1α throughout the tumour and VEGF mRNA was selectively elevated in these tumours. Both features are consistent findings in CC-RCCs with *VHL* mutations [7,18]. Only CC-RCCs exhibited elevated AM mRNA and peptide levels compared with normal kidney cortex. In a previous study, Fujita et al. [18] found very similar changes in AM and VEGF mRNAs in CC-RCCs by PCR analysis. Taken together, the findings support the concept that constitutively elevated HIF is crucial for increased expression of an array of hypoxia-controlled gene products, including AM, in CC-RCC with *VHL* mutations [7]. There are several ways that AM overexpression could affect growth of CC-RCCs. AM is a direct growth factor for certain tumour cells [19], it inhibits apoptosis [20] and promotes angiogenesis [21]. In CC-RCC, AM correlates with vascular density and could be important for tumour angiogenesis in this highly vascularized tumour type [18]. AM is significantly expressed in normal kidney and is associated with distinct nephron segments, the collecting duct system and urothelium lining the urinary tract [10]. This indicates that renal AM expression is not only controlled by hypoxia and/or HIF and this could be one reason that non-CC-RCCs also display immunohistochemical staining for AM. AM therefore appears less suited to discriminate in an 'all or none fashion' between non-CC-RCC and CC-RCC at the level of immunohistochemistry. Only quantitative measurements of AM in tumour tissue extracts document a significant difference between CC-RCC and non-CC-RCC. The significantly higher level of AM mRNA and peptide in CC-RCC prompted us to test its use as a serum marker.

Plasma AM increased acutely after excision of the kidney and tumour. There is no clearly defined organ source of AM in plasma under normal conditions [22]. The rise in plasma AM could be related to surgical manipulation of AM-rich tumours, or the adrenal gland, or to general surgical stress. Major surgical stress has been shown to elevate plasma AM concentration strongly during the operation and in the first days after surgery [23]. For this reason, we included the second measurement of

AM 4–5 weeks after surgery. After major surgery, AM stabilizes after 1 week [23]; however, the finding that AM had not decreased significantly below pre-surgery levels after 4 weeks precludes its use as a specific marker for CC-RCC. The only condition where consecutive determinations of plasma AM have been reported to reflect the presence of a tumour is phaeochromocytoma. One study [24] reported a dramatic decrease in plasma AM after adrenalectomy, whereas no difference in plasma AM was recorded in a second study of similar patients [25]. Thus there are no well-established conditions where AM could be used as a serum marker for the presence of tumour tissue at present.

In summary, our findings show that HIF-1α and the HIF-target gene products AM and VEGF are elevated in CC-RCC compared with other renal tumour types, but plasma AM is not a specific marker for CC-RCC.

ACKNOWLEDGMENTS

The study was supported by The Danish Health Science Research Council, The Danish Cancer Foundation, A. J. Andersen og Hustrus Foundation, Else Poulsen's Foundation, Jens C. Christoffersen's Foundation, and Johannes Fogh's Foundation. We acknowledge excellent technical assistance from Lis Teusch and Bodil Kristensen. We thank Professor Peter Bie for valuable advice regarding assay validation, and Anthony Carter for linguistic advice.

REFERENCES

1 Kitamura, K., Kangawa, K., Kawamoto, M. et al. (1993) Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem. Biophys. Res. Commun. **192**, 553–560
2 Martinez, A., Miller, M. J., Unsworth, E. J., Siegfried, J. M. and Cuttita, F. (1995) Expression of Adrenomedullin in normal human lung and in pulmonary tumors. Endocrinology **136**, 4099–5105
3 Nguyen, S. V. and Claycomb, W. C. (1999) Hypoxia regulates the expression of the adrenomedullin and HIF-1 genes in cultured HL-1 cardiomyocytes. Biochem. Biophys. Res. Commun. **265**, 382–386
4 Garayoa, M., Martinez, A., Lee, S. et al. (2000) Hypoxia-inducible factor 1 (HIF-1) up-regulates adrenomedullin expression in human tumor cell lines during oxygen deprivation: a possible promotion mechanism of carcinogenesis. Mol. Endocrinol. **14**, 848–862
5 Sufan, R. I., Jewett, M. A. and Ohh, M. (2004) The role of von Hippel-Lindau tumor suppressor protein and hypoxia in renal clear cell carcinoma. Am. J. Physiol. Renal Physiol. **287**, F1–F6
6 Jaakkola, P., Mole, D. R., Tian, Y. M. et al. (2001) Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science **292**, 468–472
7 Wiesener, M. S., Münchenhagen, P. M., Berger, I. et al. (2001) Constitutive activation of hypoxia-inducible genes related to overexpression of hypoxia-inducible factor1α in clear cell renal carcinomas. Cancer Res. **61**, 5215–5222
8 Eble, J. N., Sauter, G., Epstein, J. I., Sesterhenn, I. A. et al. (2004) World Health Organization Classification of Tumors. Pathology and Genetics of Tumours of the Urinary System and Male Genital Organs, IARC Press, Lyon
9 Andreasen, D., Jensen, B. L., Hansen, P. B., Kwon, T. H., Nielsen, S. and Skott, O. (2000) The α_{1G}-subunit of a voltage-dependent Ca^{2+} channel is localized in rat distal nephron and collecting duct. Am. J. Physiol. Renal Physiol. **279**, F997–F2005
10 Sandner, P., Hofbauer, K., Tinell, H. et al. (2000) Expression of adrenomedullin in hypoxic and ischemic rat and human kidneys with arterial stenosis. Am. J. Physiol. Regulatory Integrative Comp. Physiol. **286**, R942–R951
11 Lewis, L. K., Smith, M. W., Yandle, T. G., Richards, A. M. and Nicholls, M. G. (1998) Adrenomedullin(1–52) measured in human plasma by radioimmunoassay: plasma concentration, adsorption, and storage. Clin. Chem. **44**, 571–577
12 Gülberg, V., Liss, I. and Gerbes, A. L. (2000) Determination of plasma adrenomedullin concentrations with commercial radioimmunoassay kits: a note of caution. Liver Transplant. **3**, 384–385
13 Ohta, H.., Tsuji, T., Asai, S. et al. (1999) One-Step direct assay for mature-type adrenomedullin with monoclonal antibodies. Clin. Chem. **45**, 244–251
14 Zhong, H., De Marzo, A. M., Laughner, E. et al. (1999) Overexpression of hypoxia-inducible factor 1α in common human cancers and their metastases. Cancer Res. **59**, 5830–5835
15 Ehlenz, K., Koch, B., Preuss, P., Simon, B., Koop, I. and Lang, R. E. (1997) High levels of circulating adrenomedullin in severe illness: correlation with C-reactive protein and evidence against the adrenal medulla as site of origin. Exp. Clin. Endocrinol. Diabetes **105**, 156–162
16 Oehler, M. K., Fischer, D. C., Orlowska-Volk, M. et al. (2003) Tissue and plasma expression of the angiogenic peptide adrenomedullin in breast cancer. Br. J. Cancer **89**, 1927–1933
17 Ishimitsu, T., Nishikimi, T., Saito, Y. et al. (1994) Plasma levels of adrenomedullin, a newly identified hypotensive peptide, in patients with hypertension and renal failure. J. Clin. Invest. **94**, 2158–2161
18 Fujita, Y., Mimata, H., Nasu, N., Nomura, T., Nomura, Y. and Nakagawa, M. (2002) Involvement of adrenomedullin in human renal cell carcinoma. Int. J. Urol. **9**, 285–295
19 Miller, M. J., Martínez, A., Unsworth, E. J. et al. (1996) Adrenomedullin expression in human tumor cell lines. J. Biol. Chem. **271**, 23345–23351
20 Kato, H., Shichiri, M., Marumo, F. and Hirata, Y. (1997) Adrenomedullin as an autocrine/paracrine apoptosis survival factor for rat endothelial cells. Endocrinology **138**, 2615–2620
21 Zhao, Y., Hague, S., Manek, S., Zhang, L., Bicknell, R. and Rees, M. C. (1998) PCR display identifies tamoxifen induction of the novel angiogenic factor adrenomedullin by a non estrogenic mechanism in the human endometrium. Oncogene **22**, 409–415
22 Nishikimi, T., Kitamura, K., Saito, Y. et al. (1994) Clinical studies on the sites of production and clearance of circulating adrenomedullin in human subjects. Hypertension **24**, 600–604
23 Nishikimi, T., Hayashi, Y., Iribu, G. et al. (1998) Increased plasma adrenomedullin concentrations during cardiac surgery. Clin. Sci. **94**, 585–590
24 Letizia, C., Toma, G. D., Caliumi, C. et al. (2001) Plasma Adrenomedullin concentrations in patients with adrenal pheochromocytoma. Horm. Metab. Res. **33**, 290–294
25 Minami, J., Nishikimi, T., Todoroki, M. et al. (2002) Source of plasma adrenomedullin in patients with pheochromocytoma. Am. J. Hypertens. **15**, 994–997

Received 6 February 2006/2 March 2006; accepted 6 March 2006
Published as Immediate Publication 6 March 2006, doi:10.1042/CS20060030