Erythropoietin in Polycystic Kidneys

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Abstract

Erythropoietin (EPO) formation in kidneys of 18 patients with autosomal dominant polycystic kidney disease (ADPKD) was investigated. In 12 patients on hemodialysis and in 6 patients with preterminal renal failure serum, EPO was 29±7 and 16±1.5 mU/ml and hemoglobin concentrations were 11.0±0.6 and 12.7±1.2 g/dl, respectively. Cyst fluid from a total of 357 renal cysts was obtained by either in vivo aspiration or immediately after nephrectomy. The cysts contained variable concentrations of bioactive EPO from undetectable values up to 3.2 U/ml. A pronounced enrichment of EPO was observed in cysts with sodium concentrations > 100 mmol/liter, suggesting an association with proximal tubular malformations. The EPO concentrations in the cysts were neither correlated with the protein concentration nor with the oxygen pressure of the cyst fluid. Using a cDNA probe for human EPO, mRNA for EPO was localized in stroma cells of the cyst walls by an in situ hybridization technique. Our findings suggest that single interstitial cells juxtaposed to proximal tubular cysts may produce EPO independent of the oxygen pressure inside the cysts, which ameliorates the anemia during end-stage polycystic kidney disease.

Introduction

Autosomal dominant polycystic kidney disease (ADPKD)1 is a genetically determined structural and functional disorder that may lead to chronic renal failure in the adult (1). Usually patients suffering from end stage renal disease develop an anemia which results predominantly from insufficiently low erythropoietin (EPO) production by the damaged kidneys (2). Interestingly, the degree of anemia is strikingly moderated if ADPKD is the reason for renal insufficiency (3–6). Based on the observations that serum EPO levels in patients with ADPKD are on average twofold higher than in end-stage renal disease of other causes (4–6) and that renal cysts may contain erythropoietic activity (7, 8) it is assumed that an improved EPO production by the cystic kidneys is a major reason for higher hematocrit values.

To our knowledge, in vivo EPO production by cystic kidneys has not yet been systematically investigated. It was the intention of this study therefore to obtain information about the capability of polycystic kidneys to produce EPO, the intrarenal site of EPO formation, and the regulation of EPO formation in ADPKD.

Methods

Patients. Cysts from a total of 18 patients (12 male, 6 female; age, 49±2 yr; mean±SEM) were examined. All patients had history, physical, radiographic, or ultrasound, and laboratory evidence of ADPKD.

In 12 patients on regular hemodialysis, who underwent unilateral nephrectomy in preparation for renal allotransplantation, cyst fluids were aspirated within 5–10 min after surgical removal of the kidneys. Care was taken to sample cyst fluids randomly from different areas and varying depth of the kidneys. In these kidneys except two all cysts punctured (between 6 and 102 cysts per kidney) were emptied completely.

To confirm the results obtained by ex vivo puncture, in vivo cyst puncture was performed in six further patients with ADPKD before dialysis therapy had been initiated (serum creatinine 543±27 μmol/liter; mean±SEM). In these patients, cyst fluid was aspirated under ultrasound control on the occasion of therapeutic cyst sclerosing.

Cyst contents were immediately frozen for later sodium, potassium, protein, and EPO determinations. In all patients except one, peripheral venous blood samples were obtained simultaneously for determination of serum EPO levels. In five patients undergoing nephrectomy blood samples were, in addition, obtained from renal arteries and veins.

Measurement of EPO. EPO was routinely determined by RIA exactly as described previously (9). In brief, 100-μl samples plus 20 μl of 30% BSA were incubated with 100 μl rabbit antiseraum raised against human recombinant EPO for 24 h. 100 μl of tracer (8 × 10−11 mol/liter 125I-EPO; Amersham Buchler GmbH, Braunschweig, FRG) were then added and, after an additional incubation period of 24 h, separation of free and bound ligand was carried out using a second antibody technique. As a standard we used the Second International Reference Preparation of human urinary EPO (World Health Organization). Cyst fluids containing > 300 mU/ml immunoreactive EPO were randomly checked for possible EPO tracer destruction by SDS-PAGE (10) in combination with autoradiography. However, in no case did we obtain evidence for tracer destruction by cyst fluids. In some cysts EPO activity was additionally determined by the polycythemic mouse assay for EPO as described (9). Briefly, female mice (25–31 g) from the Institute of Cancer Research (ICR-strain) were exposed to intermittent (20–22 h/d) normobaric hypoxia (8% O2). On days 5 and 6 after termination of hypoxia, assay animals were injected subcutaneously with 0.5 ml of divided doses of standards or samples. On day 7, they were injected with 0.1 μCi i.p. 59Fe. 2 d later, blood was obtained by heart puncture for determination of hematocrit and iron incorporation, which was calculated on the assumption of a blood volume of 7.5% body weight. Only mice having a hematocrit of 55% or higher were used for the

1 Abbreviations used in this paper: ADPKD, adult dominant polycystic kidney disease; EPO, erythropoietin.

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calculations. The detection limits for the RIA and the bioassay for EPO were 5 and 50 mU/ml, respectively.

**Measurement of electrolytes.** Concentrations of sodium and potassium in the cyst fluids were determined by flame photometry (No. 943, Instrumentation Laboratory, Lexington, MA).

**Measurement of protein.** Protein concentration was determined in all nonhemorrhagic cysts according to Lowry et al. (11) after appropriate dilution of the samples.

**Measurement of oxygen pressure and pH value.** Po2 and pH values were determined with a blood gas analyzer (No. 1304; Instrumentation Laboratory) only in cyst fluids aspirated anaerobically by in vivo puncture.

**Histology.** Tissue from the wall of the examined cysts of patients undergoing unilateral nephrectomy was fixed in formalin, embedded in paraffin, and cut in consecutive sections. These were routinely stained by hematoxylin eosin, processed for in situ hybridization, and reserved for immunohistochemistry for Factor VIII (polyclonal mouse antibody against human Factor VIII; model AO 82; Dako Corp., Santa Barbara, CA) with the peroxidase method.

**Cloned DNA fragments and labeling.** Plasmid DNA harboring a cDNA insert of the human EPO gene was a gift of Dr. C. B. Shoemaker, Genetics Institute (Harvard, MA). It had been isolated and purified by published procedures (12). Nick-translation using 32P-ATP was done according to the method described by Rigby et al. (13). We consistently labeled 1 μg of plasmid DNA without separation of the EPO insert. Unincorporated nucleotides were separated by spundown column procedure on Sephadex G-50 columns (Pharmacia Fine Chemicals, Piscataway, NJ) as described (12). The specific activities were in the range of 107 cpm/pg and were determined by measuring aliquots dotted on NAO filters (Schleicher & Schuell, Feldbach, Switzerland).

**In situ hybridization.** 5-μm-thick paraffin sections of cyst walls were prepared as serial sections and stored at room temperature in vessels containing desiccant till use.

After spreading on organosilane-treated slides (14) dewaxing was done by immersing the slides in xylene (three times each, 10 min at 42°C). After degrading alcohol steps (abs, 70, 50, and 30% each for 2 min) the slides were washed in PBS (pH 7.4). A proteinase K incubation was included (10–15 min, 37°C, 10–50 μg/ml pretreated by self-digestion for 1 h at 37°C). This reaction was stopped by dipping the slides in PBS-0.2% glycine. Dehydrating alcohol steps (30, 50, and 70%, absolute, each for 2 min) were then incorporated. For hybridization, 300,000 cpm of the cDNA probe (corresponding to 3 ng) in 50% deionized formamide, 10% dextran sulfate, 4X SSC (standard sodium citrate: 0.15 M NaCl, 0.015 M Na2Citrate), 0.06% Ficoll, 0.06% polyvinylpyrrolidone, 0.06% BSA, 300 μg/ml sonicated salmon sperm were denatured at 100°C for 10 min, rapidly cooled on ice, and then applied to each section. A siliconized coverslip was mounted on the region provided for hybridization and sealed with Fixogum (Marabu; Ludwigsburg, FRG). The slides were then heated for 1–2 min to open secondary mRNA structures and hybridized in a wetted chamber at 42°C for 12–15 h. After this time, the coverslips were carefully removed and the slides were washed at room temperature with gentle agitation successively in 50% formamide-4X SSC, 2X SSC, and 0.2X SSC. After dehydration (see above) they were dipped in film emulsion (NTB 2; Eastman Kodak Co., Rochester, NY) diluted 1:1 with 0.6 ammonium sulfate. After air drying, the slides were exposed in black plastic boxes in the presence of silica gel at 4°C. The slides were developed with developer (D-19; Eastman Kodak) for 5 min at ~16°C. After two washes in bidistilled water, slides were fixed in rapid fixer (Eastman Kodak) and stained with hematoxylin-eosin. (Slides were stained for 8–10 min in hematoxylin, washed in running tap water for the same period, and counterstained with eosin for 30 min.) After the above degrading alcohol steps were repeated, the slides were mounted in Eukitt (Kindler; Freiburg, FRG). Internal controls using a hybridized 35S-labeled plasmid vector without EPO insert exhibited no signals. Hybridizations applying a 35S-labeled actin DNA probe exhibited weak signals distributed over all cells of the section.

**Statistics.** The Mann-Whitney test was used for comparison of groups and analysis of variance was used to determine significance values for linear regressions.

**Results**

Serum EPO levels of the patients with ADPKD included in this study ranged from 7 to 73 mU/ml, with an average of...
29±7 mU/ml (mean±SEM) in those patients on hemodialysis and from 9 to 19 mU/ml with an average of 16±1.5 mU/ml in patients with preterminal renal failure. Hemoglobin concentrations ranged from 8.8 to 14.1 with an average of 11.0±0.6 g/dl (mean±SEM) in the former and from 9.5 to 16.6 with an average of 12.7±1.2 (mean±SEM) in the latter group.

In five of the patients undergoing unilateral nephrectomy EPO concentrations could also be determined in the renal arteries and veins before extirpation. As illustrated in Fig. 1, arteriovenous concentration differences of 3 to 33 mU/ml were found.

To obtain more information about EPO production by the polycystic transformed kidneys, 344 cysts (from 12 patients) were analyzed by ex vivo and 13 cysts (6 patients) were analyzed by in vivo puncture. The incidence of different volumes and sodium concentrations for the cysts obtained by ex vivo puncture is given in Fig. 2. Most frequently, the volume of the cysts was small (≤ 0.5 ml) and the sodium concentration was high ([Na] ≥ 120 mmol/liter). The sodium content of the cyst fluids obtained by in vivo puncture fitted with this distribution. Sodium concentrations were generally inversely correlated with potassium concentrations (not shown). Sodium concentrations and volumes of cysts were not correlated (not shown). EPO concentrations in the cysts ranged from undetectable values (≤ 5 mU/ml) to 3.180 mU/ml. Most frequently, values were between 80 and 160 mU/ml (Fig. 3). Again the EPO values obtained by in vivo puncture were in accordance with the distribution shown in Fig. 3.

EPO detected in the cysts by RIA also had full biologic activity as confirmed in the polycythemic mouse assay (Fig. 4).

![Figure 3. Distribution of the EPO concentration in cyst fluids obtained by ex vivo puncture. n, absolute number of cysts within a certain range of EPO concentrations.](image1)

![Figure 4. Relationship between immunoreactivity and bioactivity of EPO found in cyst fluids.](image2)

![Figure 5. Relationship of EPO concentration in the cyst fluids to the cyst volume.](image3)

![Figure 6. Relationship of EPO concentrations in the cyst fluids to the sodium concentration. Filled symbols, cysts obtained by in vivo puncture.](image4)
Fig. 5 shows the relationship of EPO concentration to cyst volume. It is obvious that high EPO concentrations were predominantly found in small cysts. Plotting EPO against sodium concentration revealed that high EPO concentrations were found only in cysts with high sodium (Fig. 6). Also within each individual kidney, mean EPO concentrations in cysts with Na concentrations > 100 mmol/liter were higher than in cysts with Na concentrations < 100 mmol/liter (Table I). There was no correlation between EPO concentration and the protein concentration in the cyst fluid (not shown). Also, no correlation existed between either oxygen pressure or pH value in the cyst fluid and EPO concentration in those cysts aspirated in vivo (Fig. 7).

To get information about the localization of EPO-producing cells, serial tissue sections of cyst walls of three kidneys were processed for in situ hybridization for EPO mRNA, normal histology and immunohistochemistry for Factor VIII. Single stroma cells located in the cyst wall were found to contain mRNA for EPO (Figs. 8 and 9). Cyst epithelium, regular tubular cells, and glomeruli were negative. No direct evidence was obtained for a co-localization of Factor VIII and mRNA for EPO.

**Discussion**

Previous studies have found that patients with ADPKD frequently have higher values of hemoglobin and erythropoietin when compared with uremic patients suffering from noncystic renal diseases (3–6). This agrees with the concept that a higher EPO production is a major reason for the less severe anemia in these patients. It is thought that the cystic kidneys are the source of EPO in ADPKD (7, 8) and both our findings that cysts sometimes contain enormous amounts of biologically active EPO and that arteriovenous concentration differences of EPO were seen in five kidneys further support this idea. However, the intrarenal production site of EPO in the cystic kidneys is so far unknown.

Specific patterns of solute concentrations in cyst fluid corresponding to urine from various segments of normal nephrons, provide the basis for classification of cysts according to their nephron sites of origin (15, 16). With respect to sodium concentration, cysts can roughly be categorized to be of proximal (Na ≥ 100 mmol/liter), indeterminant (20 ≤ Na ≤ 100 mmol/liter) or distal origin (Na ≤ 20 mmol/liter) (17). According to this scheme 70, 15, and 15% of the cysts under our investigation were of proximal, indeterminant, and distal origin, respectively. Such a distribution has also been found in other studies (18). EPO concentrations were 204±24 (n = 251), 56±9 (n = 52), and 57±8 mU/ml (n = 54) in proximal, indeterminant, and distal cysts, respectively (P < 0.001 for proximal vs. distal or vs. indeterminant cysts). This finding indicates that EPO is significantly enriched in cysts of proximal tubular origin.

Regarding the source of the high amounts of EPO found in proximal cysts, several lines of evidence indicate that EPO levels in cysts do not result from glomerular filtration and subsequent concentration of the hormone. Microdissection and electrolyte analysis of renal cysts that have been interpreted to indicate that cyst fluid derives from glomerular filtrate (15, 16, 19, 20) suggested that cysts of all nephron sites

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**Table 1. EPO Concentrations in Gradient and Nongradient Cysts**

<table>
<thead>
<tr>
<th>Cysts</th>
<th>EPO concentrations</th>
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<tr>
<td>Nongradient cysts (Na &gt; 100 mmol/liter)</td>
<td>542±150</td>
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<tr>
<td></td>
<td>473±269</td>
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<tr>
<td></td>
<td>418±100</td>
</tr>
<tr>
<td></td>
<td>319±68</td>
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<td></td>
<td>199±16</td>
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<td>126±42</td>
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<td>77±17</td>
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<td></td>
<td>71±45</td>
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<tr>
<td>Gradient cysts (Na &lt; 100 mmol/liter)</td>
<td>32±7</td>
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<tr>
<td></td>
<td>23±3</td>
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<tr>
<td></td>
<td>30±15</td>
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<td></td>
<td>93±46</td>
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<td>120±9</td>
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<td></td>
<td>17±7</td>
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<td></td>
<td>24±4</td>
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<td>39±5</td>
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</tbody>
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Values are mean±SEM for eight kidneys in which at least three cysts of each type were aspirated. Numbers in parentheses indicate number of cysts.

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**Figure 7.** Relationship of oxygen pressure (left) and pH value (right) to the EPO concentration in the cyst fluid obtained by in vivo puncture.
are connected to nephrons. A glomerular filtration and subsequent concentration of EPO thus should result in preferentially high amounts of the hormone in distal and not proximal cysts, as found in this study. Furthermore, a more recent morphological study failed to demonstrate tubule connections in the majority of cysts, suggesting that cysts are predominantly filled by transepithelial secretion rather than glomerular filtration (21). Even assuming tubule connections of the cysts, the...

Figure 8. The same area of a polycystic kidney, stained with HE (top), in situ hybridization (middle), and immunohistochemistry for Factor VIII (bottom) ×500. The EPO-producing cells (middle) are situated between tubular cyst epithelium (arrow) and blood vessels (identified below by Factor VIII staining of the endothelial layer).
time demand for reaching high EPO levels merely by glomerular filtration is unrealistic. Calculations using a normal single nephron filtration rate of 50 ml/min in man and assuming a ratio of glomerular EPO clearance to glomerular filtration rate of 0.1, as measured for proteins of comparable size (22), reveal that, in all nine patients in whom calculations were possible, up to 21 cysts would have required > 3 yr to reach the amount of EPO measured. In 27 cysts, > 5 yr would have been necessary, and in 4 cysts > 25 yr. Finally, there was no correlation between EPO and protein concentrations in cysts, also arguing against unspecific concentration of the hormone. In consequence, a production of EPO in the region of proximal tubular cysts seems likely.

The result of the in situ hybridization indicates that the cyst wall is capable of producing EPO. But interestingly, EPO mRNA was not detected in the epithelial cells defining the cysts, but in cells located in the stroma of the cyst walls. Although we could not identify the cell type, its interstitial localization gives rise to speculation, that possible candidates are specialized endothelial, mesenchymal, and blood cells. This is a parallelism to the normal mouse kidney in which the same cell types are considered as candidates for the production of EPO (23, 24).

Irrespective of the identification of the EPO-producing cells, chiefly two possibilities arise to explain the preferential enrichment of EPO in proximal as compared with distal cysts. One explanation may rest in the relative permeabilities of gradient (proximal type) and nongradient (distal type) cysts. Thus evidence suggests that the walls of proximal cysts are more permeable to solutes than the walls of distal cysts (16, 25), indicating that substances produced in the interstitium might enter proximal cysts more readily than distal cysts. Alternatively, EPO may be preferentially produced in cells juxtaposed to proximal cysts, indicating that the proximal tubular cells are involved in the production of EPO. This latter explanation is supported by a recent study demonstrating that experimental inhibition of sodium reabsorption at the proximal tubular site, but not at any other nephron segment, significantly reduced hypoxia-induced EPO formation in mice (26).

Regardless of the mechanism resulting in particularly high EPO levels in proximal cysts, the question arises about the physiologic significance of EPO found in the cysts. Some evidence suggests that this EPO reflects overflow of EPO production in the interstitium of the diseased kidney. First, EPO in the cysts is neither related to the oxygen pressure nor to the pH value of the cyst fluid (Fig. 7). Both parameters are important physiologic determinants of EPO production in the normal kidney (27, 28). And second, a positive correlation exists between the EPO content of the cysts and the serum levels of EPO, suggesting that cyst EPO reflects the EPO production capacity of the kidney (Fig. 10). Our finding that highest EPO concentrations in the cysts were found in small cysts would be

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\text{Figure 9. EPO-producing cells in the interstitial tissues of a polycystic kidney, identified by in situ hybridization (marked by radiolabeling, \times 750).}
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\text{Figure 10. Relationship of the amount of EPO in the cysts to the respective serum EPO concentrations in patients where cysts were completely emptied and serum samples were available. Values are mean±SEM. The linear regression curve was EPO_{cyst} (milliunits) = 19.1 + 3.5 \times EPO_{serum} (milliunits/milliliter) (r = 0.80; P < 0.01).}
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in agreement with the concept that an increasing pericystic pressure causes the disappearance of EPO producing cells or a loss of their capability to produce EPO.

The question about the trigger for EPO production in the pericystic tissue remains open. The relationship between hemoglobin and serum EPO concentrations for the patients with ADPKD strikingly differs from the normally found inverse relationship of anemic patients with intact renal function (6, 29, and own observations). In contrast, it appears from previous studies (4, 6) as if the hemoglobin concentration and EPO are positively correlated, suggesting an autonomous rather than a regulated EPO production. A genetical determination of EPO production in association with ADPKD seems not very likely, because also solitary cysts have been found to contain EPO and to be accompanied with elevated serum EPO levels (7, and own unpublished data). It seems reasonable therefore to assume that the stimulus for EPO production is related to the alteration of the functional architecture of peritubular tissue.

Acknowledgments

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