Tumor Cells Are the Site of Erythropoietin Synthesis in Human Renal Cancers Associated With Polycythemia

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One to five percent of human renal cell carcinomas are associated with polycythemia. It is generally assumed that polycythemia results from the secretion of erythropoietin (Epo) by the malignant cells. However, there is no direct proof supporting this hypothesis. Three patients with typical renal adenocarcinoma and polycythemia were studied. All three exhibited high Epo serum levels as measured by radioimmunoassay (RIA). A strong Epo signal was observed on Northern blot analysis of total RNA extracted from the renal tumors. The Epo message seemed to be of normal size and no Epo gene rearrangement was observed with the restriction enzymes tested. Using the in situ hybridization technique, a significant labeling was constantly observed on the tumor cells. Immunohistochemical studies showed that these tumor cells, known to be of tubular origin, were labeled by an anti-cytokeratin antibody and therefore were of epithelial nature. Thus, this study demonstrated that malignant cells of tubular origin were able to produce Epo constitutively, whereas in the mouse hypoxic kidney, peritubular cells (probably capillary endothelial cells) were the major site of Epo synthesis.

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Erythropoietin (Epo), the hormone that controls erythropoiesis in mammals, is produced mainly by the kidney. An increased Epo production has been observed in malignant diseases associated with polycythemia. These rare diseases include renal cell carcinomas, nephroblastomas, liver cell carcinomas, and hemangioblastomas of the cerebellum. So far, the nature of the cells that synthesize Epo in human cancers associated with polycythemia remains unknown. Using in situ hybridization, we report that tumor cells of epithelial origin are the site of Epo production in three patients with renal adenocarcinoma and polycythemia.

MATERIALS AND METHODS

Patients. The characteristics of these three patients are depicted in Table 1. All three exhibited high Epo serum levels. The tumor sizes were 7 cm for patient 1 and 10 cm for patients 2 and 3. Tissues from renal tumors and nontumoral adjacent kidneys were obtained after nephrectomy, frozen immediately, and stored in liquid nitrogen for Northern blot analysis and in situ hybridization. Human fetal liver (20 weeks) was used as a control in Northern blot analysis. As an additional control, we used normal and tumoral kidney tissues from a patient nephrectomized for renal cell carcinoma without polycythemia. In two patients, peripheral blood leukocytes (PBL) were collected for DNA isolation.

Probe. The probe used was a 642 base pair KpnI-BglII insert of Epo monkey complementary DNA (cDNA). This probe was 32P-labeled using the random priming method. RNA extraction and Northern blot analysis. Frozen tissues from the three tumors, nontumoral neighboring kidneys, and human fetal liver were crushed to a powder and homogenized in 4 mol/L guanidine thiocyanate. Total RNA was extracted by the method of Chirgwin et al. After glyoxal denaturation, 20 μg of total RNA were electrophoresed and transferred to gene screen membranes, and the blotted RNAs were hybridized to the 32P-labeled Epo probe (specific activity: 4 × 106 cpm/μg).

In situ hybridization. Five-micrometer thick sections of unfixed frozen tumor tissues from three patients were prepared. They were fixed in 4% formaldehyde in phosphate buffer saline (PBS) (0.1 mol/L, pH 7.4) for 20 minutes and dehydrated in alcohols. The procedure for in situ hybridization has been described previously. Briefly, tissue sections were hybridized under a sealed coverslip for 24 hours at 42°C in 15 μL of a solution containing 50% deionized formamide; 4X standard saline citrate (SSC), pH 6.2; 0.02% Ficoll; 0.02% polyvinylpyrrolidone; 0.02% bovine serum albumin; 10% dextran sulfate; 2 μg/mL yeast tRNA (Sigma, St Louis, MO); 400 μg/mL salmon sperm DNA (Sigma); 40 μg/mL herring sperm DNA (Sigma); 10 mol/L dithiothreitol; and 0.2 ng/mL of the 35S-radiolabeled probe denatured at 100°C for 2 minutes (specific activity: 2 × 108 cpm/μg).

Slides were then washed twice at 46°C with gentle agitation in 40% formamide-4X SSC for 15 minutes, followed by three washes in 2X SSC at 60°C for 15 minutes, and then three times in 2X SSC at room temperature for 15 minutes. Sections were then dehydrated in alcohols and covered with Kodak NTB2 emulsion (Rochester, NY) for autoradiography. After 7 to 30 days of exposure, the slides were developed in Kodak D19, fixed with Kodak A44, and stained with hematoxylin and eosin.

Control procedures were performed to assess the specificity of the in situ hybridization labeling: (1) treatment of tissue sections with 50 μg/mL ribonuclease A (type III, Sigma) in 2X SSC for 30 minutes at 37°C before hybridization with the Epo probe; (2) hybridization with a 35S-labeled pBR vector used as a nonrelevant probe; and (3) to assess the background level and the probe specificity, normal kidney, nonscreting renal cell carcinoma, and Epo-secreting carcinoma sections were processed on the same slide and hybridized with the Epo probe.

Immunohistochemistry. Frozen tumor tissue sections were fixed in cold acetone for 10 minutes, rehydrated in PBS, and incubated for 30 minutes with a monoclonal anti-human cytokeratin antibody used at a 1:100 dilution (KL1; Immunotech, Marseille, France), a monoclonal anti-human vimentin antibody used at a 1:10 dilution (Dakopatts, Copenhagen, Denmark), or a monoclonal anti-human von Willebrand factor antibody used at a 1:5 dilution (Dakopatts). After washing in PBS, antibodies were shown using the peroxidase-anti-peroxidase technique.

DNA extraction and Southern blot analysis of the renal tumors. High molecular weight DNAs were extracted from renal tumors and peripheral blood leukocytes of patients 1 and 2. DNAs were digested with restriction endonucleases as recommended by the manufactur-
turer. Digested DNA samples (10 μg) were transferred to nylon membranes after electrophoresis and hybridized to the ^3P-labeled Epo probe under stringent conditions.

RESULTS

**Messenger RNA (mRNA) expression in tissue extracts.** All three tumors exhibited a strong signal when Northern blots of total RNA were hybridized with the Epo probe (Fig 1). Signal intensity differed from one patient to another, but the size of the Epo mRNA seemed to be identical to human fetal Epo mRNA (1.6 kilobases [kb]). An upper band weak in intensity could be seen in most lanes, which probably corresponded to a nonspecific hybridization with 28S ribosomal RNA. Purification of poly (A)+ RNA was not attempted due to the small size of the tumoral samples obtained. In the nontumoral renal tissue adjacent to the tumor mass, no Epo message could be detected, although similar amounts of total RNA were transferred to the filters, as demonstrated by hybridization with the actin probe (Fig 1, lane 4).

**Localization of Epo-producing cells by in situ hybridization.** In all tumor sections, a significant hybridization was constantly observed in the tumor cells, whereas the stroma constituted of extracellular matrix and vessels was at the background level (Fig 2, A through C). The intensity of the signal observed within individual tumoral cells was heterogeneous, the significance of which is unknown. Ribonuclease-treated sections and sections hybridized with a pBR plasmid were negative. Furthermore, hybridization performed on normal kidney and nonsecreting renal cell carcinoma sections failed to detect any signal, whereas the Epo-secreting renal carcinoma sections on the same slide were positive.

**Immunohistochemical studies.** To further determine the nature of these Epo-producing cells, tumor sections were first labeled with an anti-cytokeratin antibody. As shown in Fig 3A, most of the tumor cells exhibited a strong cytoplasmic labeling indicating the epithelial nature of these cells. With an anti-vimentin antibody, the tumor cells were positive in 1 of 2 tumors tested (data not shown). In contrast, tumor cells were negative after incubation with the anti-von-Willebrand antibody, which strongly labeled endothelial cells located outside the tumoral nests (Fig 3B).

**Search for an Epo gene rearrangement in tumor DNAs.** Two patients were studied. To eliminate a possible polymorphism, each tumor DNA was compared with normal DNA prepared from the patient PBL. As shown in Fig. 4, no rearrangement was observed with the four restriction enzymes tested.

**DISCUSSION**

The occurrence of polycythemia in renal cell carcinoma is a rare (1% to 5%) but not fortuitous event. It is probably related to an increase in Epo production. Furthermore, cell lines of renal origin producing Epo in culture have been reported. In this report we studied three patients with typical renal adenocarcinomas associated with polycythemia. In the three patients, Epo serum levels determined by a sensitive and accurate RIA method were found to be significantly increased. High levels of Epo mRNA in the tumor extracts on the one hand and absence of Epo expression in the normal neighboring tissue on the other hand led us to two conclusions. (1) In these polycythemic patients, kidney carcinomas were a major site of Epo production. When looking at the three patients as a whole, the sizes of the tumors were similar and relatively large. However, given the variability of the stromal content and the amount of necrosis within a tumor, it is difficult to directly relate the size of a tumor to the amount of Epo produced. In fact, there was no obvious correlation between hematocrit, Epo serum level, Epo mRNA signal on Northern blot analysis, and in situ hybridization data, except for patient 3 where almost all parameters were high. (2) This Epo production seemed to be constitutive and was probably no longer physiologically regulated because the patients were polycythemic. However, no measurement of oxygen tension has been performed in the tumoral tissues. We cannot exclude that profound local hypoxia commonly described in solid tumors could trigger Epo production. Apparently, this abnormal Epo production was not due to a DNA rearrangement in the vicinity of the Epo gene as judged by Southern blot analysis. Furthermore,
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Fig 2. In situ hybridization of tumor tissue sections from patients 1 and 3 using the 35S-labeled Epo probe. (A) Patient 1: Many tumor cells are labeled (arrows), whereas the stroma (stars) is clearly negative (bar, 50 μm). (B and C) Patient 3: A very strong signal is observed in most of the tumor cells (arrows). In the stroma (stars), the cells are negative. (B) Bar, 50 μm. (C) Bar, 20 μm.

the size of the tumor Epo mRNA seemed to be identical to that of the fetal liver and the previously reported size of Epo mRNA in humans.

The main result of this study is the demonstration that the Epo-producing cells are the tumor cells in renal adenocarcinomas associated with polycythemia. As demonstrated with cytokeratin, these cells are of epithelial nature. In one case they also express vimentin, a common marker of mesenchy-
mal cells. However, coexpression of cytokeratin and vimentin has been described as a consistent feature in renal cell carcinomas. The tubular origin of human renal adenocarcinomas is well-established. These tumors are currently supposed to derive from proximal tubular cells. Endothelial cells were shown to be outside the tumor cell areas by an anti-von Willebrand factor antibody. These results are in apparent contradiction with previous publications by our

![Image](image.png)

**Fig 3.** Immunohistochemistry in tumor tissue sections from patient 3, using the peroxidase-antiperoxidase (PAP) technique. (A) Tumor cell cytoplasms are labeled with the anti-cytokeratin antibody (arrows) (bar, 20 μm). (B) The anti-von Willebrand factor antibody, an endothelial marker, clearly localizes the vascular endothelial cells (arrows) outside the tumor cells (bar, 30 μm).

**Fig 4.** Southern blot analysis of digested DNAs from patients 1 and 2: a and b are DNAs isolated from patient 1 PBL and renal tumor, respectively, c and d are DNAs isolated from patient 2 PBL and renal tumor, respectively. The migration of Hind III-digested phage λ size markers is indicated in kilobases on the left of the figure.

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group\textsuperscript{16} and others\textsuperscript{31} showing that in the mouse anemic kidney, peritubular cells (most probably endothelial cells) were the site of the Epo production. To explain this discrepancy, we could hypothesize that the physiologic Epo-producing cells would be different in humans and mice. However, this seems unlikely considering the high level of conservation over evolution of the physiologically regulated erythropoietic system.

This Epo production by renal tumoral cells deriving from proximal tubular cells raises the question about the possible physiological Epo production by these tubular cells. A low constitutive Epo production by the tubular cells cannot be excluded if the level of expression is below the threshold of detection of the in situ hybridization technique.

However, data have shown that although renal cell carcinomas may be derived from proximal tubular cells,\textsuperscript{32} they do not necessarily express the same proteins, as evidenced by the vimentin expression in some renal cell carcinomas while it is never present on normal proximal tubular cells. In fact, it is well-known that oncogene cell transformation is often associated with abnormal gene expression; eg, we previously described an erythroleukemic cell line producing Epo,\textsuperscript{33} whereas normal erythroblasts apparently do not. Finally, the main conclusion that can be drawn from this study is that the polycythemia exhibited by some patients with renal adenocarcinomas is due to an Epo production by cancer cells within the tumor mass.

**ACKNOWLEDGMENT**

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**REFERENCES**


