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Erythropoietin stimulates proliferation of human renal carcinoma cells

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Erythropoietin stimulates proliferation of human renal carcinoma cells.

Background. We reported recently that normal human, rat, and mouse tubular cells express authentic erythropoietin-receptors (EPO-R) through which EPO stimulates mitogenesis. The present study examines whether EPO could elicit such a proliferative and thereby potentially detrimental response in cells of human renal-cell carcinoma (RCC).

Methods. Nephrectomy samples were screened from patients with RCC (one chromophilic, two clear cell) as well as cell lines of human (Caki-2, 786-0) and mouse (RAG) renal adenocarcinomas for expression of EPO-R transcripts and protein. Cells were further tested for specific ^{125}I-EPO binding and mitogenic response to EPO.

Results. Authentic EPO-R transcripts and protein (approximately 72 kD) were detected in renal tumors and cell lines. Tumors showed low-level EPO expression, while cell lines did not. In cells, specific ^{125}I-EPO binding to a single class of EPO-R (apparent K_d 1.3 to 1.4 nmol/L, B_{max} 2.2 to 2.6 fmol/mg protein) was observed. EPO stimulated cell proliferation dose dependently, and the individual mitogenic effects of either EPO or 10% newborn calf serum were markedly amplified when both were coadministered.

Conclusion. These data are the first to demonstrate, to our knowledge, that human RCCs express EPO-R message and protein and that receptor activation stimulates their proliferation in vitro. If these mitogenic effects of EPO are also operative in patients with RCC, endogenous EPO or its administration for the treatment of anemia could potentially hasten proliferation of renocellular malignancies.

Recently, we reported that all regions of normal human, rat, and mouse kidney, as well as glomerular mesangial, proximal tubular, and medullary collecting duct cells express authentic erythropoietin receptors (EPO-R) whose activation stimulates cell proliferation in vitro [1]. In erythroid progenitor cells, EPO stimulates cell proliferation, inhibits apoptosis, and induces cell differentiation in a stage-specific manner [reviewed in 2, 3]. Although the in vivo functions of EPO-Rs in the kidney have yet to be determined, our recent observations suggest that EPO may act as a cytokine in renal cells.

Most types of human renal-cell carcinomas (RCCs) are derived from proximal tubular cells [4, 5], which carry functional EPO-Rs [1]. If mitogenically active EPO-Rs continue to be expressed in malignantly transformed proximal tubular cells, endogenous or administered EPO could hasten tumor growth. Possible detrimental effects of EPO on tumor growth may be further mediated by its known angiogenic activity [6–10]. Renal cell carcinomas are commonly hypervascular, and experimental interventions that enhance or inhibit angiogenesis are known to accelerate or retard tumor growth, respectively [11, 12].

In general, only 5% or less of patients with RCC develop polycythemia caused by paraneoplastic overproduction of EPO, while approximately 35% develop significant anemia [13, 14], which may be treated with recombinant EPO. Physiological EPO production is stimulated by tissue hypoxia and occurs primarily in peritubular fibroblasts situated in the renal cortical labyrinth [2, 15–17]. Rare clinical cases of EPO-secreting RCCs, composed of malignantly transformed proximal tubular cells, have been described [18–24]; their cells, when grafted into athymic nude mice, are tumorigenic and produce polycythemia caused by constitutive EPO secretion. Activation of renal EPO-Rs might thus occur in RCCs by both endocrine and paracrine pathways. Furthermore, the coexpression of EPO and its receptor in the same cell could allow autocrine stimulation and could potentially lead, as seen in some hematological malignancies, to autocrine transformation [25].

The prognostic significance of increased EPO production in patients with RCC is unclear. Janik et al first suggested that increased EPO production by RCCs may mark interleukin-2 (IL-2)/α-interferon (IFN)–responsive tumors [26]; they reported that IL-2/α-IFN therapy resulted in significant responses in five patients with metastatic RCC and polycythemia. They concluded, however, that in a larger series of patients, only 15% of those with

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polycythemia responded to this therapy, the same response rate obtained in nonpolycythemic RCC patients, and furthermore, all five patients who responded initially suffered subsequent relapses and metastatic disease. A later in vitro study suggested that EPO-producing cells of RCC exhibited higher susceptibility to lysis by LAK cells [27]. In a subsequent study, Ljungberg, Rasmuson, and Grankvist suggested that increased serum EPO levels in the absence of polycythemia, observed in a surprisingly high 33% of their 165 patients, carried a worse prognosis and indicated a higher incidence of progressive metastatic disease [28]. On the other hand, a prospective study on 49 patients with RCC found essentially no evidence for paraneoplastic EPO overproduction, and also failed to detect EPO expression in various established cell lines of human RCC [29].

The impact of administered EPO on the growth of RCC is also uncertain. A case report on a single patient with metastatic RCC suggested that high doses of EPO caused a complete remission of all lesions [30]. In a later study of 20 such patients given similar EPO treatment, one achieved complete remission. Thirteen showed partial or minor responses, and the remaining six were unaffected [31]. Since publication of these reports, no rigorously controlled studies have confirmed the suggested positive effects of EPO in patients with RCC. The observation, finally, that EPO administration in an anemic patient with multiple myeloma may have caused further malignant transformation resulting in plasma cell leukemia called into question the safety of EPO treatment for patients with EPO-R expressing myeloma cells [32]. Thus, considered together, the available data are unable to yield conclusive evidence regarding the prognostic significance of (*1*) EPO production by RCCs and (*2*) their response to EPO administration.

Advanced stages of RCC continue to carry a poor prognosis and are often associated with EPO-responsive anemia [11, 14]. EPO acts, as we demonstrated [1], as a mitogen in normal renal cells. It stimulates proliferation and migration of vascular endothelial cells [9, 10], and it augments angiogenesis [6–8]. Because these cellular responses to EPO may collectively promote tumor growth and because these actions may be enhanced further by high endogenous EPO production or by EPO administration, in the present study we examined (*1*) whether RCC and malignant renal tubular cell lines express EPO-R and (*2*) whether receptor activation stimulates their proliferation.

We found intense expression of EPO-R message and protein in human renal cell carcinoma and in established cell lines of renal cancer. Activation of the EPO-R stimulated cell proliferation in vitro. These observations are compatible with the notion that EPO administration to patients with RCC could accelerate tumor growth. Until it is demonstrated that EPO lacks such trophic effects in vivo, we suggest that treatment of RCC patients with EPO should be carried out with some degree of caution.

METHODS

Human kidney tissue and cell lines of renal carcinoma

Nephrectomy specimens from three male patients (ages 54, 61, and 62) with confirmed RCC were obtained intraoperatively with Internal Review Board (IRB) approval. Anatomical localization of tumors within the kidney was determined, and the tumor and adjacent normal-appearing tissues were immediately placed into chilled phosphate-buffered saline (PBS), rinsed free of blood, and frozen in liquid nitrogen or fixated (10% formaldehyde) for histologic examination as reported [33]. Tissue samples were stored at −70°C until RNA was extracted. Representative portions of the renal tumors and adjacent normal kidney tissues were processed for histologic examination, paraffin embedded, sectioned, and stained with periodic acid-Schiff stain. Tumor histology was recorded photographically.

Two established human renal carcinoma cell lines, Caki-2 and 786-0, were examined (ATTC, Rockville, MD, USA). Both are derived from human renal adenocarcinomas. Caki-2 cells have no mutation of the von Hippel-Lindau (VHL) tumor suppressor gene, while 786-0 cells do [34]. In addition, a murine renal adenocarcinoma cell line, RAG, derived from BALB/cd strain of mice was studied (ATTC). Caki-2 cells were grown in McCoy's 5a medium and 10% newborn calf serum (NCS; Hyclone, Logan, UT, USA), 786-0 cells in RPMI 1640 and 10% NCS, and RAG cells in Dulbecco's modified Eagle's medium (DMEM) and 10% NCS. All cultures were incubated at 37°C in 5% CO_2/air and grown to high subconfluence.

As controls, normal human proximal tubular cells (HCT; Clonetics Corp., San Diego, CA, USA) at passage 2 were grown to high subconfluence in a 1:1 mixture of Dulbecco's modified Eagle's medium and F12 containing 1 × ITS (insulin, transferrin, selenium; Sigma, St. Louis, MO, USA), epidermal growth factor (EGF; 10 ng/mL), T3 (4 pg/mL) and 10% NCS at 37°C in 5% CO_2/air.

mRNA isolation, reverse transcription-polymerase chain reaction, and gel electrophoresis

Total RNA was isolated from tissues and cultured cells by an acid guanidinium-thiocyanate-phenol-chloroform method [35], using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). For the reverse transcription-polymerase chain reaction (RT-PCR) reactions, 1 μg of total mRNA was reverse transcribed (M-MLV reverse transcriptase; GIBCO, Grand Island, NY, USA), and the resulting cDNAs were subjected to 35 cycles of the PCR. Amplification of the examined cDNAs was linear up to 35 PCR cycles for all primer sets used in

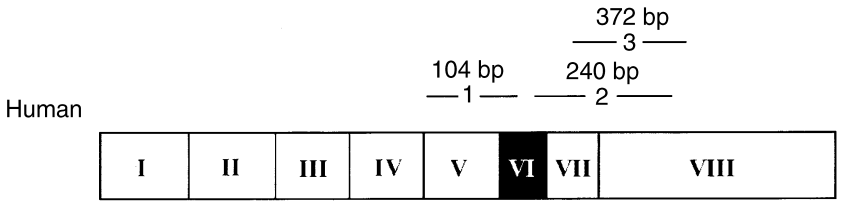

Fig. 1. Schema of the mRNA that encodes the erythropoietin receptor (EPO-R) [36, 38]. It is composed of eight exons; exons I through V represent the exoplasmic domain (N-terminus), exon VI, the single transmembrane domain, and exons VII and VIII, the cytoplasmic domain (C-terminus). Regions of the human EPO-R that are bound by the three polymerase chain reaction (PCR) primers (1 through 3) and the size of the obtained PCR products are indicated (not to scale).

this study. For human samples, three sets of PCR primers were chosen, each specific to a different domain of human EPO-R cDNA (Fig. 1). Their individual sequences and respective positions within the coding sequence are as previously reported [1, 36].

For mouse EPO-R cDNAs, two sets of primers were used for PCR amplification of homologous sequences as previously reported [1, 37, 38].

For the detection of human EPO mRNA, the following set of PCR primers was used [39]: sense, 24-mer, 5′ position 335, 5′-ATC ACG ACG GGC TGT GCT GAA CAC-3′, and antisense, 24-mer, 3′ position 600, 5′-GGG AGA TGG CTT CCT TCT GGG CTC-3′, yielding a 289 bp PCR product containing exons II through V.

As internal controls, human and mouse β-actin mRNAs derived from the same kidney tissues and cultured cells were identically reverse transcribed and PCR amplified. The primers used for human β-actin cDNA were as previously reported [1], yielding a 279 bp product. All primer pairs for EPO-R and EPO cDNA were chosen to span introns (Fig. 1), thus facilitating control for contamination by genomic DNA. All cell and tissue extracts were subjected to the PCR reaction once with and once without initial reverse transcription.

All PCR products were size fractionated on 2.5% NuSieve agarose and 1% agarose gel (FMC Bioproducts, Rockland, ME, USA), stained with ethidium bromide, and band locations were recorded by photographing them under ultraviolet light. Size standards of DNA were run in parallel (pBR322 DNA MSP I digest; New England Bio Labs, Beverly, MA, USA).

Specific erythropoietin binding to cells

Cells were grown to subconfluence, as described previously in this article, and mechanically harvested. They were then washed at 4°C for three minutes with 0.5 mol/L NaCl and 0.25 mol/L acetic acid, pH 2.5, in order to remove EPO bound to the cell surface [40] and washed twice more with PBS, pH 7.3. Binding assays, using ^{125}I-rhEPO (specific activity 948 Ci/mmol; Amersham, Arlington Heights, IL, USA) and unlabeled human EPO (recombinant human EPO; Amgen, Royal Oaks, CA, USA) were carried out as previously reported [1]. To obtain K_d and B_{max}, both linear Scatchard [41] and nonlinear (GraphPad Software, Inc., San Diego, CA, USA) regression analyses of the equilibrium-binding data were performed. The time course of ligand binding was determined by incubating ^{125}I-rhEPO at a concentration of 620 pmol/L with approximately 1×10^6 cells at 15°C for four hours. To test whether specific EPO binding has a linear correlation with the number of cells used, increasing numbers of cells from 0 to 9×10^6 were incubated with ^{125}I-rhEPO (620 pmol/L) for three hours at 15°C both in the absence and the presence of 200-fold excess unlabeled EPO.

Immunological detection of erythropoietin receptor protein

Freshly resected tumor and adjacent normal tissue and cultured Caki-2 and 786-0 cells were examined. The latter were grown to high subconfluence in 75 cm^2 tissue culture flasks, washed twice with ice-cold PBS, and collected by scraping and centrifuging. The pellet was frozen at −70°C. Cell membrane protein extracts from kidney tissues and cells were prepared as previously reported [1, 42]. These were then mixed 1:1 with 40 μL 2 × Laemmli's sample buffer and heated at 100°C for four minutes prior to electrophoresis on 9% sodium dodecyl sulfate (SDS)-polyacrylamide gel, as previously reported [43, 44]. The separated protein bands were then electrophoretically transferred to Hybond-P membrane (Amersham). A 1:1000 dilution of the primary antibody (affinity purified, polyclonal rabbit anti–EPO-R antibody, specific for human or mouse and rat; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in blocking buffer was then added. Preparations were further incubated, and after three washes in TBS-T, the second antibody was added (1:1000 dilution of an horseradish peroxidase-conjugated anti-rabbit IgG; Santa Cruz Biotechnology), followed by a one-hour incubation at room temperature. After four washes in TBS-T, the protein bands were made visible, using the enhanced chemiluminescence detection method (ECL; Amersham), and recorded on radiographic film as previously reported [1, 43].

In addition, HeLa (ATTC) and HCD-52 cells were used as negative and positive EPO-R–expressing controls, respectively [1]. Western blots from the latter were

generated, following prior immunoprecipitation, as previously reported [1].

The specificity of anti–EPO-R antibody binding by tumor and cell membrane protein extracts was further assessed by reacting the primary blots, prepared as described previously in this article, with a 1:1000 dilution of primary antibody that had been neutralized (17 hours at 4°C) with a 10-fold (by mass) excess of EPO-R antigen (C-terminal peptide; Santa Cruz Biotechnology). The remaining steps of the immunodetection protocol were then performed as described previously in this article.

Erythropoietin secretion

Erythropoietin secretion by logarithmically growing (at 24, 48, and 72 hours) and from quiescent Caki-2 and 786-0 cells (after 24 hours in serum-free media) was determined using a commercial enzyme-linked immunosorbert assay (ELISA) (ALPCO, Windham, NH, USA). The utilized batches of 10% NCS (Hyclone, Logan, UT, USA) were also tested for EPO levels. The lower detection limit for this EPO assay is 10 mU/mL.

Cell proliferation

All tested cell lines were seeded at 1×10^4/well in 96-well plates and allowed to attach overnight in appropriate serum-containing media. Cells were rendered quiescent by incubation in serum-free media for 24 hours. Recombinant human EPO alone, at incremental concentrations, or NCS (10%) alone or in combination with various EPO doses, was then added, and the incubation was continued for 24 or 48 hours as previously reported [1]. At these times, media were removed and replaced with fresh media (without phenol red and serum) containing 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Thiazolyl blue; Sigma) and incubated at 37°C for an additional three hours. After this, the media were removed, and 100 μL of 0.04 N HCl in isopropanol were added to each well in order to solubilize the blue MTT reduction product formed in viable cells. The absorbance was read on a microplate reader at 570 nm with background subtraction at 650 nm. In addition, cell proliferation was assessed by hemocytometer counting of trypsinized cells. There was excellent linear correlation ($r = 0.99$) between cell counts and results obtained by the MTT assay.

Data reporting and statistical analysis

All data on human renal tumors and individual cell lines are representative of at least three to six replications or independent experiments, respectively. Data are reported as means ± SE. Differences between data means were analyzed for statistical significance ($P < 0.05$) using Student's *t*-test for paired and independent populations as well as analysis of variance [45]. Apparent K_d and B_{max} values for ^{125}I-EPO binding were derived by both linear Scatchard [41] and nonlinear (GraphPad Software, Inc., San Diego, CA, USA) regression analyses of the equilibrium-binding data.

RESULTS

EPO receptor and EPO expression in human renal cell carcinoma

Figure 2 shows the histology of a renal carcinoma resected from one of the three adult patients whose renal tumors were screened for EPO-R expression (discussed in the **Methods** section). This cortical tumor is of the granular or chromophilic cell type with solid growth pattern, high vascularity, and of nuclear grade 3 [46, 47]. The other two tumors were of the clear-cell type. The two clear-cell adenocarcinomas contained occasional small cysts and were also highly vascularized.

Figure 3 shows ethidium bromide-stained agarose gels of EPO-R PCR products obtained from the tumor shown in Figure 2. The bands in lanes 3, 4, and 5 represent the expected 104, 372, and 240 bp PCR products of the human EPO-R cDNA (Fig. 1). The first lane shows DNA size standards and lane 2 the 249 bp β-actin PCR product, used as internal control. The EPO-R PCR products derived from the other two tumor specimens and from adjacent normal renal tissue, as we previously reported [1], were indistinguishable from those depicted in Figure 3.

Both the renal carcinomas and normal renal cortical tissue showed only a low-level EPO message. All three patients were anemic at the time of diagnosis (mean HCT 32 ± 2%), and none of the patients were polycythemic during the year preceding the resection of their tumors.

EPO receptor and EPO transcripts in malignant renal cells

To determine further whether malignant renal epithelial cells express EPO-R mRNA, two defined cell lines that are derived from human RCCs were examined. This was important since the EPO-R transcripts that were found in renal tumor tissue could be expressed, as we demonstrated recently [1], by nonepithelial cells such as glomerular mesangial cells or by vascular endothelial or intravascular erythroid progenitor cells. EPO-R expression in the murine RCC cell line RAG was also examined.

Figures 4 and 5 show ethidium bromide-stained agarose gels of EPO-R PCR products obtained from Caki-2 and 786-0 cells, respectively. The bands in lanes 4, 5, and 6 in both figures represent the expected 104, 372, and 240 bp PCR products of the human EPO-R cDNA (Fig. 1) generated from Caki-2 and 786-0 cells, respectively. The first lanes show DNA size standards and lanes 2 the 249 bp β-actin PCR product, used as internal control. In lane 3, the reverse transcriptase step was omitted, and no PCR products were detected. Neither Caki-2 nor 786-0

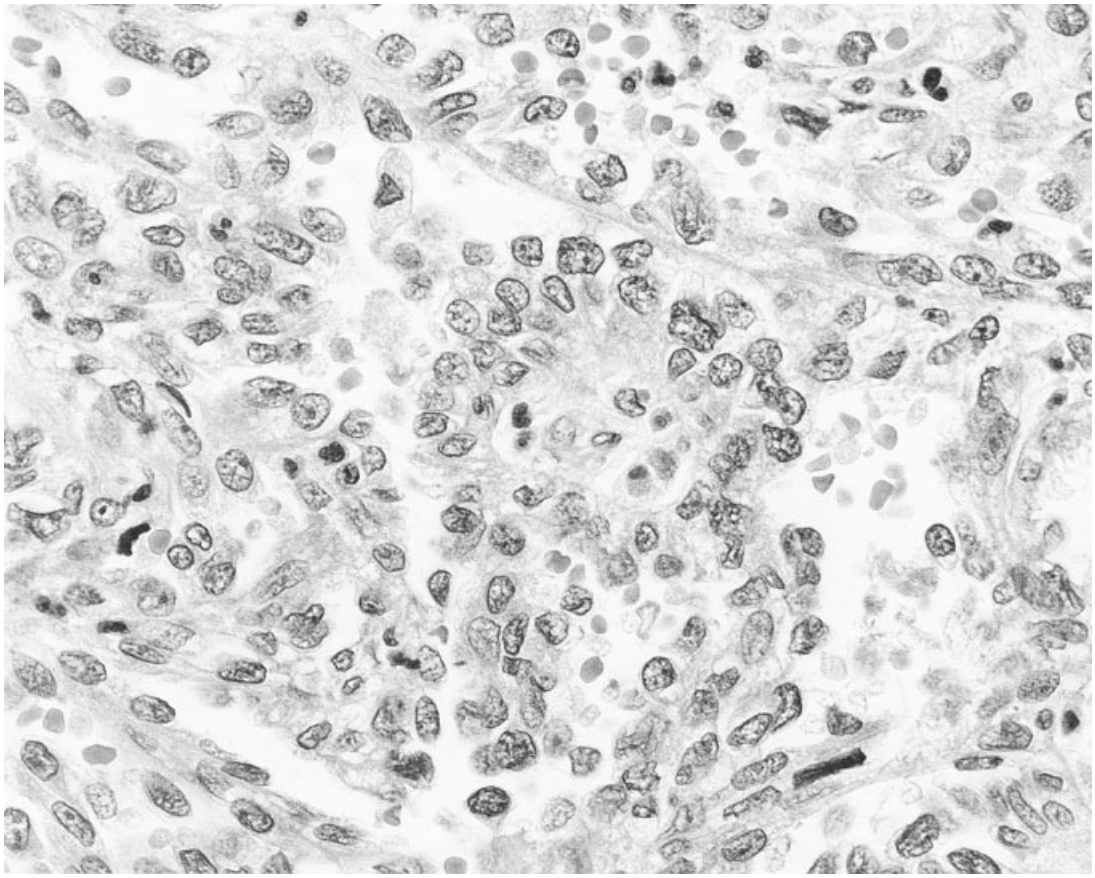

Fig. 2. Histology of human renal cell carcinoma. Granular or chromophilic cell type, vascular tumor of nuclear grade 3 [46, 47]. Original magnification ×250, periodic acid-Schiff stain.

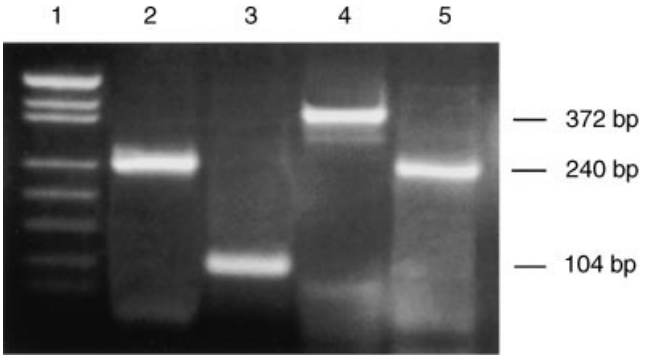

Fig. 3. EPO-R transcripts of human renal cell carcinoma. Ethidium bromide-stained gel shows in lane 3 the expected 104 bp EPO-R PCR product (1. primer set in Fig. 1), in lane 4 the 372 bp (3. primer set), and in lane 5 the 240 bp PCR (2. primer set) from the renal carcinoma depicted in Figure 2. The first lane shows DNA size standards, and lane 2 the 249 bp β-actin PCR product, used as internal control. The EPO-R transcript patterns of adjacent normal renal cortex (1) and of the other two RCCs, both of clear cell type, were identical to that depicted here. EPO transcripts were detectable at low intensity in both normal and tumor tissues (data not shown).

cells expressed EPO mRNA. In RAG cells, EPO-R mRNA was detected in a pattern indistinguishable from that obtained in non-neoplastic murine proximal tubular cells (data not shown) [1]. None of the cell lines secreted detectable amounts of EPO into the media.

Specific EPO binding to cell surface and immunologic detection of EPO-R protein

In human renal cancer cells, Caki-2, and normal HCTs, as well as in murine RAG cells, specific binding of ^{125}I-EPO to cell surface EPO-R was examined. Specific binding of ^{125}I-EPO in all cell lines occurred to a single class of receptors with intermediate affinity (Caki-2, apparent K_d 1.4 ± 0.2 nmol/L, B_{max} 2.2 ± 0.3 fmol/mg protein, $N = 3$; HCT, apparent K_d 1.1 ± 0.1 nmol/L, B_{max} 1.6 ± 0.2 fmol/mg, $N = 3$; RAG cells, apparent K_d 1.3 ± 0.3 nmol/L and B_{max} 2.6 ± 0.2 fmol/mg, $N = 3$), and ligand binding became saturated within approximately 1.5 hours. Nonlinear and Scatchard analyses yielded comparable results. There was excellent linear correlation ($r = 0.99$) between specific ligand binding in tested cell lines and cell numbers. It is of note that specific binding of

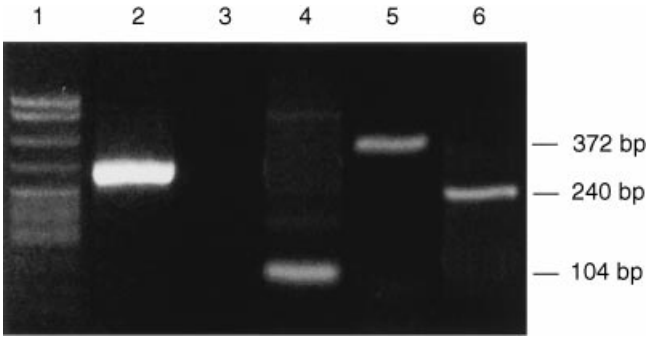

Fig. 4. EPO-R and EPO expression in Caki-2 malignant renal cells. Ethidium bromide-stained gel shows in lanes 4, 5, and 6 the expected EPO-R PCR products (104, 372, and 240 bp) as defined in Figure 1 and as detected in RCC (Fig. 3). Lane 1 shows DNA size standards. Lane 2 shows the 249 bp β-actin PCR product, used as internal control, and in lane 3, the reverse transcriptase step was omitted. Caki-2 cells do not express EPO mRNA.

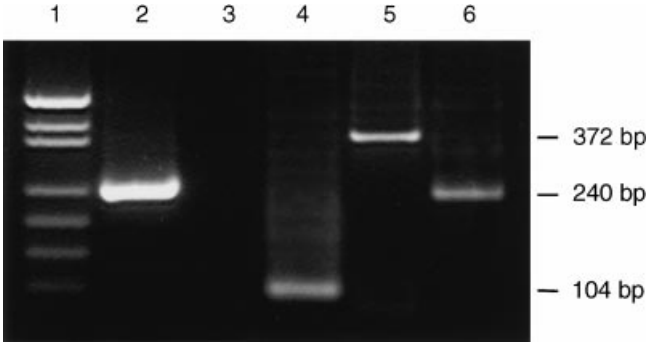

Fig. 5. EPO-R and EPO expression in 786-0 malignant renal cells. Ethidium bromide-stained gel shows in lanes 4, 5, and 6 the expected EPO-R PCR products (104, 372, and 240 bp) as defined in Figure 1 and as detected in RCC (Fig. 3) and Caki-2 cells (Fig. 4). Lane 1 shows DNA size standards. Lane 2 shows the 249 bp β-actin PCR product, used as internal control, and in lane 3, the reverse transcriptase step was omitted. Caki-2 cells do not express EPO mRNA.

^{125}I-EPO in murine proximal tubular cells occurred with significantly higher affinity (K_d approximately 96 pmol/L) than in the cells examined here [1].

Western blots for EPO-R protein were generated using membrane protein extracts of resected tumor tissue, Caki-2 and 786-0 cells (Fig. 6A). Lane 1 on Figure 6A shows molecular weight standards, and lanes 2 (tumor tissue; Fig. 2), 3 (Caki-2) and 4 (786-0), depict immunoreactive EPO-R protein bands, all with an approximate molecular mass of 72 kD. When anti–EPO-R antibodies were first neutralized with an excess of specific antigen (C-terminal EPO-R peptide; **Methods** section), no EPO-R–specific immunoreactivity was detected. Figure 6B shows control Western blots from EPO-R–negative HeLa cells, lane 1, and from EPO-R expressing HCD-57 cells, lane 2. Lane 3 depicts molecular weight standards. As previously demonstrated by Sawyer and Hankins [48] and others [3],

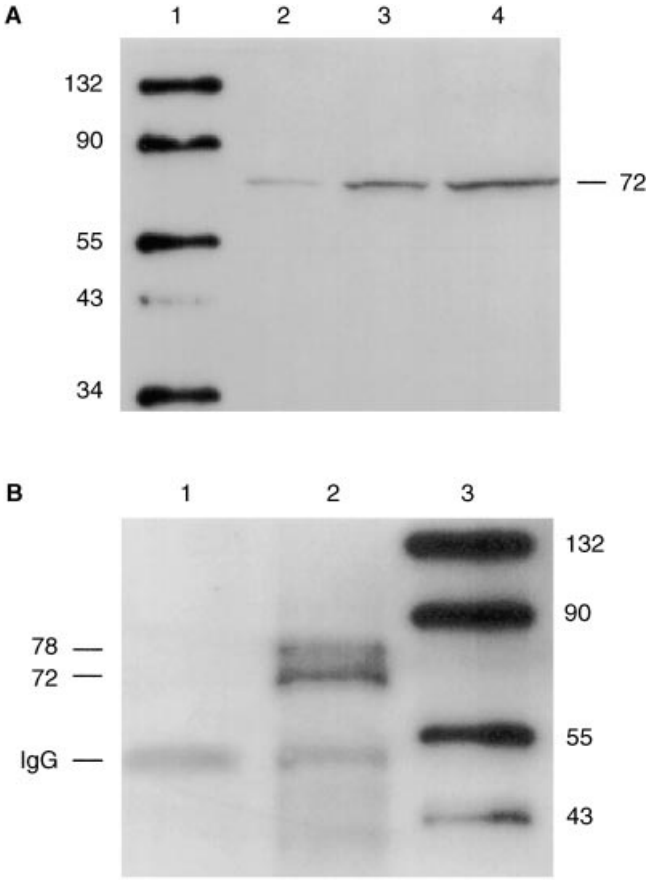

Fig. 6. Immunodetection of EPO-R protein in RCC, Caki-2, and 786-0 cells. (*A*) Western blots for EPO-R protein were generated using membrane protein extracts of resected tumor tissue, Caki-2, and 786-0 cells. Lane 1 shows molecular weight standards, and lanes 2 (tumor tissue, Fig. 2), 3 (Caki-2), and 4 (786-0) depict immunoreactive EPO-R protein bands with an approximate molecular mass of 72 kD, respectively. When anti–EPO-R antibodies were first neutralized with an excess of specific antigen (C-terminal EPO-R peptide; **Methods**), no EPO-R–specific immunoreactivity was detected. (*B*) Control Western blots generated following immunoprecipitation of cell membrane preparations from EPO-R negative HeLa cells (lane 1) and EPO-R–expressing HCD-57 cells (lane 2). The latter express EPO-R protein with molecular masses of 72 and 78 kD [48]. The 72 kD form is also found in RCC cells (Fig. 6A). Lane 3 depicts molecular weight standards.

HCD-57 cells express functional EPO-R with molecular masses of 72 and 78 kD. The predominant 72 kD form is also present in RCC cells (Fig. 6A).

Mitogenic action of EPO in malignant renal cells

Figure 7 A, C, and E demonstrate that EPO, dose dependently and significantly, stimulates cell proliferation in serum-deprived Caki-2, 786-0, and RAG cells. The maximal proliferative response in 786-0 cells was achieved with 1 to 5 U/mL of EPO, while progressively higher EPO doses caused further proliferation of Caki-2 cells. These responses were, as in normal renal cells, most prominent at 48 hours following EPO administration [1]. Addition of 10% NCS to "quiescent" Caki-2 (Fig. 7B), 786-0 (Fig. 7D), and RAG (Fig. 7E) cells consistently

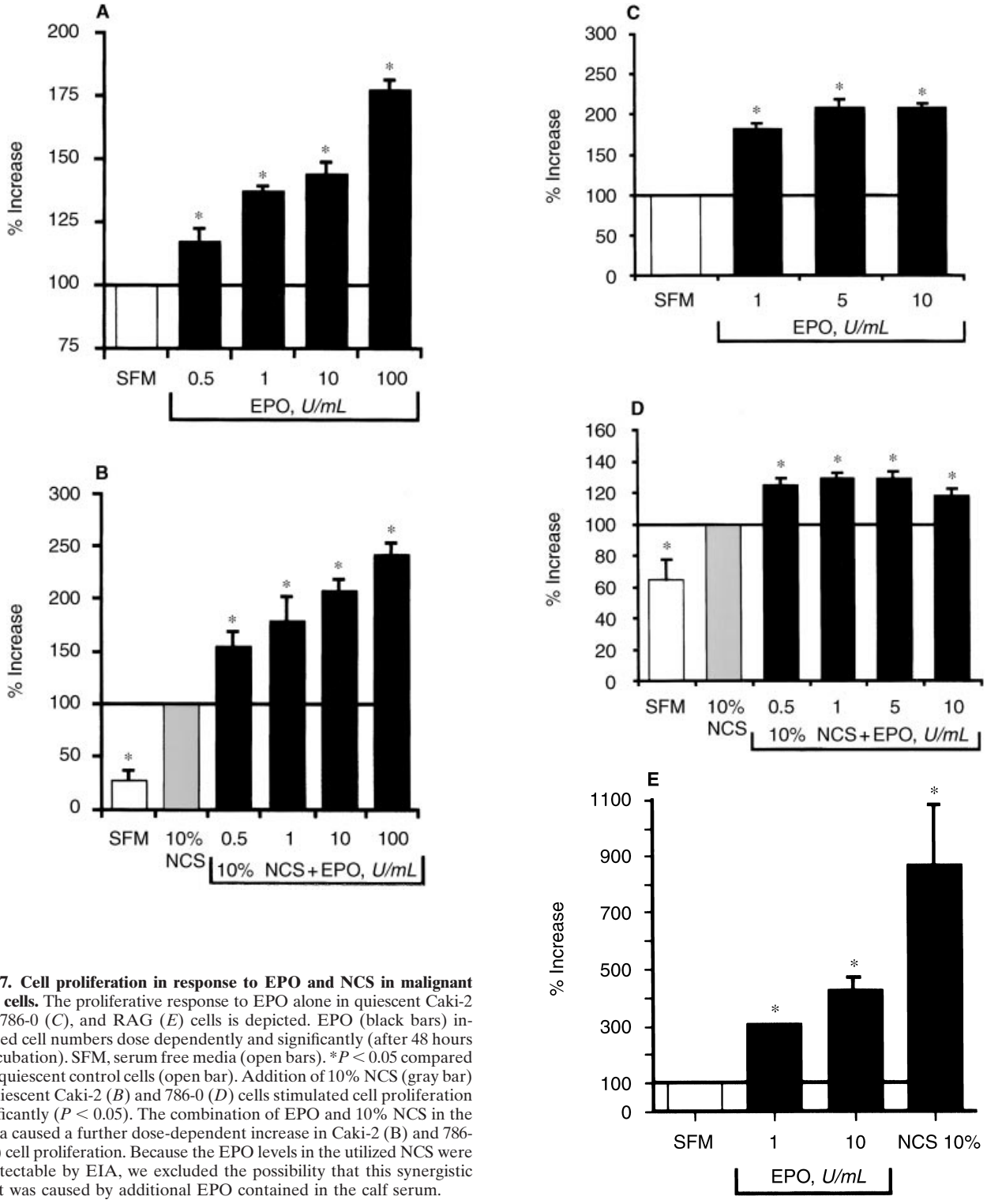

Fig. 7. Cell proliferation in response to EPO and NCS in malignant renal cells. The proliferative response to EPO alone in quiescent Caki-2 (*A*), 786-0 (*C*), and RAG (*E*) cells is depicted. EPO (black bars) increased cell numbers dose dependently and significantly (after 48 hours of incubation). SFM, serum free media (open bars). $^*P < 0.05$ compared with quiescent control cells (open bar). Addition of 10% NCS (gray bar) to quiescent Caki-2 (*B*) and 786-0 (*D*) cells stimulated cell proliferation significantly ($P < 0.05$). The combination of EPO and 10% NCS in the media caused a further dose-dependent increase in Caki-2 (B) and 786-0 (D) cell proliferation. Because the EPO levels in the utilized NCS were undetectable by EIA, we excluded the possibility that this synergistic effect was caused by additional EPO contained in the calf serum.

stimulated cell proliferation by approximately 1.6- to 8-fold ($P < 0.05$), and this response served as positive control. Figure 7 B and D illustrate that the combination of EPO and 10% NCS in the media caused a further dose-dependent increase in Caki-2 and 786-0 cell proliferation. The response patterns of both cell lines were again similar to those obtained in serum-free media (Fig. 7 A, C) in that the proliferative response of 786-0 cells plateaued with 0.5 to 1 U/mL of EPO, while incremental EPO doses led again to further increases in Caki-2 cell

proliferation. Since the EPO levels in the utilized NCS were undetectable by EIA (<10 mU/mL), we excluded the possibility that this additive effect was caused by additional EPO contained in the calf serum.

DISCUSSION

Our data demonstrate clearly that human RCC tissue and cell lines both express authentic EPO-R mRNA and protein. The expression intensity, although not specifically quantitated, resembles that seen in normal kidney or control proximal tubular cells [1]. While specific EPO binding to the cell surface EPO-R occurs with somewhat lower affinities in both the malignant and the primary HCT cell lines than in erythroid progenitor or mouse proximal tubular cells ($K_d \sim 1.1$ to 1.6 nmol/L vs. $\sim$ 96 pmol/L, respectively) [1], maximal ligand binding, B_{max}, in malignant and normal human cells is greater. EPO alone stimulates cell proliferation of quiescent Caki-2, 786-0, and RAG cells; the mitogenic response in the human cell lines is amplified when EPO is combined with 10% NCS. Undetectable EPO levels in the utilized calf serum indicate that this additive effect must result from synergism between EPO and other growth factors or cytokines that are contained in NCS. Potential candidates for this synergism with EPO are insulin, insulin-like growth factor-I, EGF, transforming growth factor-α (TGF-α), TGF-β, hepatocyte growth factor, platelet-derived growth factor, basic fibroblast growth factor, IL-6, and probably others [14, 49–56]. Receptors for most of these have been identified in cell lines derived from RCCs, including Caki-2 and 786-0 cells, and their respective activation was shown to stimulate or modify cell proliferation.

Damen et al reported recently that hyper-responsiveness to EPO in murine hemopoietic cells that express C-terminally truncated EPO-Rs is only obtained when cells are incubated with fetal calf serum that is known to contain IGF-I [57]. Since IGF-I has an EPO-sparing effect in patients with anemia of chronic renal failure [58] and since it is a renotropic mitogen [59], we searched for but were unable to demonstrate such synergism between EPO and IGF-I in cultured tubular cells (unpublished preliminary data). Although we did not specifically search for C-terminal truncations of the EPO-R, we found, using the third primer set as defined in Figure 1, a largely intact C-terminal domain that encodes, in part, a negative growth-regulatory site [3]. Truncations, however, that include the C-terminus itself, and immediately adjacent sequences would not have been detected by our analysis. Accordingly, C-terminal EPO-R truncations that may contribute to the observed synergistic effects of EPO and serum cannot be entirely ruled out. Whether the serum-induced increase in cellular responsiveness to EPO could result from amplification of EPO-R expression, as occurs in serum-stimulated transformed hemopoietic cells [60], is not known. It is equally unexplored whether the high proliferative index found in RCCs is associated with an analogous increase in EPO-R expression that results in magnified responsiveness to EPO and thus proliferation of tumor cells, and it is also unclear whether the very low proliferative index of normal tubular cells is associated with decreased EPO-R expression [1], which, in turn, could explain why normal kidneys are largely unresponsive to EPO in vivo.

Since activating mutations of the EPO-R were found to cause transformation of hematopoietic cells [61], the question arises whether this also occurs in RCCs. And finally, pathologically elaborated EPO in RCCs may conceivably be mutated, as was shown in hepatoma cells [62], which may also cause changes in ligand-binding kinetics and thus alter postreceptor events.

Anagnostou et al demonstrated previously that EPO stimulates proliferation and migration of vascular endothelial cells [9, 10], and Carlini, Reyes, and Rothstein [6] and Yasuda et al [8] showed that EPO augments angiogenesis in vitro and in vivo, respectively. It has also been demonstrated that normal renal vessels and mesangial cells express vasoactive EPO-R [42]. This implies that endothelial cells of renal tumor vessels likewise express EPO-Rs through which EPO could stimulate angiogenesis and thus advance tumor growth. The central role of angiogenic factors in progression of RCC is well recognized. More than 70% of patients with sporadic clear cell carcinoma show inactivating mutations of the *VHL* gene [11, 14], whose wild-type gene product acts to suppress tumor growth through negative control of angiogenic factors such as vascular endothelial growth factor, TGF-α, and TGF-β [34]. Whether EPO, via its angiogenic activity, can play an analogous role in the growth of RCCs awaits examination.

Taken together, it remains to be investigated which of the hypothetical mechanisms considered previously in this article actually apply to the observed responses elicited by EPO and NCS in the present in vitro study. Despite these unanswered questions, our data suggest that EPO possesses the potential to alter the growth of those RCCs whose neoplastic epithelial and vascular cells express functional EPO-Rs.

The 786-0 cell line, unlike Caki-2 cells, lacks the wild-type *VHL* tumor suppressor gene [34]. Although the EPO-R expression patterns in both cell lines were similar (Figs. 4–6), their mitogenic responses to EPO and NCS were different (Fig. 7). In 786-0 cells, relatively low doses of EPO (0.5 to 1.0 U/mL), both in serum-free media and in combination with 10% NCS, elicited maximal, although modest cell proliferation. In contrast, Caki-2 cells responded with further increases in proliferation to progressively higher doses of EPO. An explanation for these differences in growth response is not currently available. It is furthermore unknown whether the pres-

ence or lack of wild-type VHL protein has any direct bearing on EPO-R expression and activation. It is of note in this context that the lack of the *VHL* gene product, a tumor suppressor, leads in patients with RCC to enhanced proliferative and angiogenic activity. The growth response pattern of Caki-2 cells is compatible with maintained or up-regulated EPO-R expression, while that of 786-0 cells may reflect ligand-induced EPO-R down-regulation. The possibilities have not been investigated.

One larger clinical study of patients with RCC suggested that elevated serum EPO levels signified a poor prognosis [28]. In vitro studies on two RCC-derived cell lines showed neither a growth stimulatory nor an inhibitory effect of EPO [63]. Another study pertinent to this question demonstrated that grafts of EPO-secreting RCC cells in athymic nude mice were less tumorigenic than non-EPO–secreting cells [22]. The latter observation suggests that the secretion of EPO failed to increase the aggressiveness of these tumors, arguing against the presence of an autocrine loop for EPO. On the other hand, if EPO-Rs were absent or down-regulated, these tumors would obviously not be expected to respond to EPO. It therefore follows that a complete understanding of these data will depend on whether or not EPO-secreting RCC cells actually express functional EPO-R.

The cell lines tested in the present study did not express EPO mRNA and did not secrete EPO into the media. Numerous other established cell lines of RCCs also tested negative for EPO expression [29]. This eliminates the possibility of an EPO-driven autocrine growth pattern in the former cells. It is of interest that an autocrine loop for EPO secretion was detected in Hep3B cells [64], a human hepatoma cell line that secretes EPO in response to hypoxia or cobalt and expresses cell surface EPO-R. Autocrine activation of EPO-Rs in these cells was found to boost EPO secretion. Whether EPO released by these cells altered cell proliferation was, however, not examined. A proliferation-enhancing autocrine loop for EPO in EPO-R–expressing leukemic cells has been described [25, 65].

Two earlier clinical reports suggested that EPO administered to a single patient with metastatic RCC [30] and to another patient out of a group of 20 with RCC [31] caused tumor regression, while in the remaining patients EPO slowed disease progression or was ineffective. The proposed beneficial effect of EPO in these patients is in direct conflict with our present observations, however. Surprisingly, these optimistic accounts of EPO therapy were never followed up by rigorously controlled studies. In fact, it is possible that the positive responses ascribed to EPO were either spontaneous remissions or induced by prior IL-2 therapy [31] as cases of both possibilities have been reported [11, 14]. In our own study of five patients with RCC, IL-2 therapy caused complete remission in one, a partial remission in one, and no effect in the other three [66]. None of our patients presented with polycythemia, and EPO levels were not determined. Although it was suggested that EPO expression by RCC cells may indicate heightened responsiveness to IL-2/α-IFN therapy [26], this impression was not confirmed when larger numbers of patients were evaluated.

In conclusion, our present data do not support the notion that high ambient EPO levels have a curative effect in RCC. Rather, our data provide significant initial evidence to suggest that EPO administration to patients with RCC has the potential of modifying tumor growth by stimulating cell proliferation and possibly angiogenesis. We suggest that these responses are mediated by activation of EPO-Rs that are expressed in malignant renal tubular cells and that have not been described previously. These observations in neoplastic tubular cells also provide further insight into possible in vivo functions of renal EPO receptors. We finally believe that our data, although obtained in vitro, should make clinicians aware of the possibility that EPO administration may adversely affect patients with RCC. Conclusive evidence regarding EPO's potential significance as a tumor-promoting factor will, however, depend on the utilization of in vivo RCC models and on rigorous outcome studies conducted in RCC patients who are treated with EPO.

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