Autocrine Stimulation by Erythropoietin (Epo) Requires Epo Secretion

By Jean-Luc Villeval, Maria Teresa Mitjavila, Isabelle Dusanter-Fourt, Françoise Wendling, Patrick Mayeux, and William Vainchenker

Erythropoietin (Epo) autocrine stimulation has been implicated in erythroblastic leukemia. To examine whether this stimulation could occur intracellularly, we developed Epo autocrine models of stimulation in the human pluripotent UT-7 cell line. Retroviral expression of Epo totally abolished the growth factor requirement of UT-7 cells. Autonomous proliferation was not cell density-dependent and occurred at a unicellular level, showing a genuine autocrine mode of stimulation. Total blockage of Epo secretion induced by the endoplasmic reticulum-retention amino acids Lys-Asp-Glu-Leu (KDEL) signals in 11 lines prevented autonomous proliferation, whereas a leaky retention system, observed in 3 other lines, resulted in limited autocrine stimulation without true long-term autonomous proliferation. Production of Epo, in contrast to KDEL-modified Epo, induced reductions in Epo binding, Epo receptor (EpoR) mRNA, and phosphorylation levels similar to those induced by the addition of exogenous Epo to the parental cell line. In addition, autonomous growth and survival were inhibited by the addition of Epo-neutralizing antibodies, affording evidence that autocrine stimulation through EpoR activation takes place on the cell surface. Finally, phenotypic analysis of the virus-infected clones indicated that Epo production did not change the differentiative capacities of UT-7 cells. All these data show that Epo autocrine stimulation is dependent on Epo secretion and takes place on the cell surface. From all analyzed parameters, the effects of Epo autocrine stimulation and those of exogenously added Epo appear to be identical.

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From INSERM U.362, Institut Gustave Roussy, Villejuif; and INSERM U.363, Hôpital Cochin, Paris, France.

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Address reprint requests to Jean-Luc Villeval, PhD, INSERM U.362, Institut G. Roussy, 39, rue C. Desmoulins, 94805 Villejuif, France.

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Epo autocrine stimulation does not allow the same rapid transformation.

One hypothesis explaining these differences is related to the localization (intracellular or extracellular) of the stimulation. It has been shown that EpoR/EP55 complexes and disulfide-linked mutant cEpoR dimers accumulate in the ER.18,24 It was proposed, albeit controversially, that these events were involved in the induction of the disease.25 Similarly, we have previously shown that, in one case of human erythroleukemia, autonomous cell growth was inhibited by Epo mRNA antisense oligonucleotides but not by Epo-neutralizing antibodies, suggesting the occurrence of an intracellular mode of stimulation.19 In fact, it is still unknown whether an Epo autocrine stimulation may truly occur intracellularly or whether it has the same consequences on cell differentiation and proliferation as an extracellular stimulation.

To investigate this phenomenon, we have developed models of extracellular and intracellular autocrine stimulation using the human Epo-dependent cell line, UT-7.26 Our results show that retroviral expression of Epo leads to an autonomous growth of the cell line affecting EpoR expression and phosphorylation. This Epo autocrine stimulation is inhibited by the presence of Epo antiserum. In contrast, retroviral phosphorylation. This Epo autocrine stimulation is inhibited by the presence of Epo antiserum. In contrast, retroviral expression of Epo that is modified with the ER-retention system amino acids Lys-Asp-Glu-Leu (KDEL) does not modify the proliferative or differentiative capacities of the cells and did not affect the EpoR metabolism. These data strongly suggest that Epo autocrine stimulation requires the interaction of Epo with its receptor on the cell surface.

MATERIALS AND METHODS

Cell cultures. Parental and virus-infected UT-7 cells27 were cultured in Iscove’s modified Dulbecco’s medium (Iscove; GIBCO/ BRL, Grand Island, NY) or in methylcellulose semisolid medium, as previously described.27 Media contained 10% fetal calf serum (FCS) (Eurobio, Paris, France), recombinant human (rh) GM-CSF (2.5 ng/mL; Genetics Institute, Cambridge, MA), or rhEpo (2 U/mL; Amersham, Les Ulis, France). Cultures were performed at 37°C in a fully humidified atmosphere of 5% CO₂. The DaE7 cell line (Epo-dependent clone from the Da-1 cell line,28,29 obtained from Dr. H. Nomura, Chugai Pharmaceutical Co., Shizuoka, Japan) was cultured in IMDM containing 10% FCS in the presence or absence of GM-CSF, and the DaE7 cell line was transfected with the psi-CRIP cell line to produce viruses able to infect the human UT-7 cells.

The DaE7 cell line was transfected with an adenoviral vector containing the EpoR cDNA sequence. The cells were infected with the adenoviral vector at a multiplicity of infection of 100 and the infected cells were collected and expanded. The psi-CRIP cell line was transfected with an adenoviral vector containing the EpoR cDNA sequence. The cells were infected with the adenoviral vector at a multiplicity of infection of 100 and the infected cells were collected and expanded. The psi-CRIP cell line was transfected with an adenoviral vector containing the EpoR cDNA sequence. The cells were infected with the adenoviral vector at a multiplicity of infection of 100 and the infected cells were collected and expanded. The psi-CRIP cell line was transfected with an adenoviral vector containing the EpoR cDNA sequence. The cells were infected with the adenoviral vector at a multiplicity of infection of 100 and the infected cells were collected and expanded. The psi-CRIP cell line was transfected with an adenoviral vector containing the EpoR cDNA sequence. The cells were infected with the adenoviral vector at a multiplicity of infection of 100 and the infected cells were collected and expanded. The psi-CRIP cell line was transfected with an adenoviral vector containing the EpoR cDNA sequence. The cells were infected with the adenoviral vector at a multiplicity of infection of 100 and the infected cells were collected and expanded.

For cultures involving limiting cell dilution, UT-7 cells were seeded at 0.5 cell in 20 μL per well in five 60-microwell plates in the presence or absence of GM-CSF. After a 1-hour sedimentation, the number of plated cells per well was recorded. Colony formation was observed after 8 days of incubation at 37°C.

Microwell assays29 were performed in duplicate by adding 200 cells (from the DaE7 or the UT-7 cell line) in 10 μL volume of IMDM plus 10% FCS to serial twofold dilutions of (1) cell-conditioned media or cell lysates (Epo assays), (2) Epo standards (for Epo responsiveness studies), or (3) Epo neutralizing antibodies (for cell growth inhibition studies). Viable DaE7 or UT-7 cells were counted after 2 or 4 days of incubation at 37°C, respectively. For antibody experiments, control cultures were performed in the presence of GM-CSF or an excess of Epo (30 U/mL). The titer of the anti-Epo antibodies30 was determined by adding Epo-stimulated cells (1 U/mL) to serial twofold dilutions of the antibodies. The anti-Epo IgG fraction from the rabbit Epo antiserum was purified using diethylami-noethyl-Affi-Blue gel (Bio-Rad, Richmond, CA) chromatography.

Retroviral vector construction and production (Fig 1). All viruses are based on the MPZen2 vector containing a 3' myeloproliferative sarcoma virus long terminal repeat (LTR).13 To construct the ZenNeo vector, a HindIII-EcoRI fragment encoding the neomycin resistance (Neo) gene was inserted into the polylinker site of the MPZen2 plasmid DNA. To construct the ZenEpo retrovirus, a neo- mycin resistance gene controlled by the Simian virus 40 (SV40) early promoter-enhancer (SV), was inserted into the ClaI site of the previously described MPZenEpo virus.14 To construct the ZenEpoK virus, the BamHI-BamHI fragment of the cynomolgus monkey Epo cDNA sequence was removed from the MPZenEpo virus and subcloned into the M13 mp8 vector. Oligonucleotide-directed mutagenesis (Amersham) was performed using an oligonucleotide encoding a 12-amino acid sequence introducing the KDEL sequence in 5' of the Epo cDNA stop codon. This modification was confirmed by DNA sequencing. The modified Epo cDNA (EpoK) was inserted 5' of an SV40 early promoter-enhancer into a Bluescript vector (Strategene, La Jolla, CA). The cassette encompassing the modified Epo gene preceded by the SV40 promoter was placed, using ClaI linkers, into the ClaI site of the ZenNeo vector.

The psi-2 packaging cell line was plated at 10^6 cells in 10 mL, 1 day before transfection. The vector plasmid DNAs (10 μg) were transfected into these cells by the calcium phosphate precipitation method. One day after transfection, the psi-2 cells were plated in 96 wells (4 × 24 well plaques, 1 mL/well) in the presence of 400 μg/mL G418 (GIBCO/BRL). After 15 days, G418-resistant clones (around 200) were isolated and expanded. For the ZenEpo and ZenEpoK vector-transfected psi-2 cells, conditioned media from confluent cultures were assayed for Epo production, and only the 10 best Epo-producer clones were tested for virus production. The relative viral titers of the psi-2 clones were determined by comparing their ability to convert FDC-P1 cells into G418-resistant FDC-P1 cells. Briefly, 2 × 10^5 FDC-P1 cells were cocultivated in a 1:1 ratio with irradiated (20 Gy) psi-2 cells from each clone. After 2 days, nonadherent FDC-P1 cells were collected, washed, and plated at 200 cells/dish in conventional agar cultures in the presence of the absence of G418 (1.5 and 2 mg/mL). The percentage of G418-resistant cells was calculated after 1 week of culture.

UT-7 cell infection procedure. Supernatants from the best virus-producer psi-2 clones were used to infect the amphotropic retrovirus-packaging psi-CRIP cell line to produce viruses able to infect the human UT-7 cells. Fresh filtered supernatants (0.22 μm, 2 mL) were collected and expanded. For the ZenEpo and ZenEpoK vector-transfected psi-2 cells, conditioned media from confluent cultures were assayed for Epo production, and only the 10 best Epo-producer clones were tested for virus production. The relative viral titers of the psi-2 clones were determined by comparing their ability to convert FDC-P1 cells into G418-resistant FDC-P1 cells. Briefly, 2 × 10^5 FDC-P1 cells were cocultivated in a 1:1 ratio with irradiated (20 Gy) psi-2 cells from each clone. After 2 days, nonadherent FDC-P1 cells were collected, washed, and plated at 200 cells/dish in conventional agar cultures in the presence of the absence of G418 (1.5 and 2 mg/mL). The percentage of G418-resistant cells was calculated after 1 week of culture.

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Fresh filtered supernatants (0.22 μm, 2 mL) from confluent bulk G418-resistant psi-CRIP cultures were used to infect 5 × 10^6 UT-7 cells in the presence of GM-CSF and polybrene (4 μg/mL) for 3 hours. Aliquots of these filtered supernatants were tested for virus titers and the presence of replication-competent virus. After infection, UT-7 cells were washed and cultured without G418 in the presence of GM-CSF. After 2 days, one-third of the bulk-infected UT-7 cell culture was plated in 20 methylcellulose semisolid medium culture dishes, in the presence of G418 (1 mg/mL) and GM-CSF.
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Fig 1. Structure of the retroviruses. The base MPZen vector\textsuperscript{16} contains splice donor (SD) and acceptor (SA) sites and 3' hybrid LTRs, encompassing large U3 LTR sequences of the myeloproliferative sarcoma virus (MPSV). Sites of transcriptional initiation are indicated with arrows at the LTR and internal SA sites and 3' hybrid LTR, encompassing Zen EpoK retrovirus, the oligonucleotide encoding SV40 early region promoter (SV) sequences. In the abbreviated as follows: C, cDNA stop codon. Restriction endonuclease sites are represented as follows: C, Cla I; E, EcoR I; H, Hind I1.

for cloning. After 7 days of incubation, G418-resistant colonies were isolated and amplified in liquid cultures in the presence of G418 and tested for replication-competent virus secretion and proviral integration.

Replication-competent virus production was tested by the mobilization assay using the 3T3-BAG cell line and by the reverse transcriptase assay.\textsuperscript{12}

Epo assays. Epo concentrations were measured using both a radioimmunologic assay (RIA; Epo-TRAC kit; Sorin Biomedica, Saluggia, Italy) and a biologic assay.

Biologic assays were performed using the microwell assay\textsuperscript{30} with the UT-7 cell line or the murine DaE7 cell line. Epo concentrations were calculated from rhEpo standards. Because these cell lines respond to several growth factors,\textsuperscript{32} the presence of Epo was ascertained by inhibiting biologically active samples with an Epo antisera (1,000 times diluted, a dilution inhibiting 1 U/mL of Epo). RIA was standardized from biologically active monkey Epo.

Cell lysates were prepared from each virus-infected UT-7 clone and parental cells. Cells were washed twice with IMDM and brought to a final cell concentration of 20 x 10\textsuperscript{6} cells/mL in the lysis buffer (20 mmol/L Tris [pH 8.0] solution containing 10% FCS, 1 mmol/L EDTA, 2 mmol/L phenylmethylsulfonyl fluoride, 1% aprotinin, and 0.5 mmol/L leupeptin). Cells were lysed with 10 cycles of freezing and thawing and were centrifuged at 12,000g for 30 minutes at 4°C, and cell lysates were collected from the supernatants. Epo standards for the calculation of Epo concentrations in cell lysates were parental UT-7 cell lysates in which known amounts of Epo were added to the lysis buffer.

Cell conditioned media were prepared from 2 x 10\textsuperscript{6} cells/mL cultured in IMDM, 10% FCS, in the presence of GM-CSF and collected 2 to 4 days later when the cell concentrations were around 5 to 8 x 10\textsuperscript{6} cells/mL.

The number of Epo molecules was evaluated assuming that 1 mU represented 2.6 x 10\textsuperscript{10} molecules. This calculation is based on a specific activity of 125,000 U/mg of protein and a molecular weight of 18 kD for Epo.\textsuperscript{11}

RNA analysis. Total cellular RNA was isolated according to the method of Chomczynsky and Sacchi\textsuperscript{19} or Gough\textsuperscript{19} from infected clones or the UT-7 parental cell line. Northern blot analysis (20 μg of RNA per lane) was performed as previously described,\textsuperscript{13} using formaldehyde/agarose gel electrophoresis and transfer to Hybond C-Extra membranes (Amersham). cDNA hybridization probes were, for the Epo probe, a 600-bp BamHI-BamHI Epo cDNA fragment from the ZenEpo plasmid DNA; for the Neo probe, a 1,350-bp HindIII-EcoRI Neo cDNA fragment from the ZenNeo plasmid DNA; and for the EpoR probe, an approximately 1,000-bp BglII EcoRI fragment from an hEpoR cDNA plasmid.\textsuperscript{33} RNA integrity was checked by visualizing 28S and 18S ribosomal RNA stained in ethidium bromide.

The polymerase chain reaction (PCR) technique was applied to Epo mRNA as previously described,\textsuperscript{13} with an antisense 3' PCR primer CTTCCAGGCAATAGAATAC (nucleotide [nt] 237 to nt 217) and a sense 5' primer GCCCCCACACGCTCATCTGT (nt 82 to nt 102).

DNA blot hybridization. DNA from UT-7–infected cells was isolated by overnight digestion with proteinase K (0.3 mg/mL) in a 50 mmol/L Tris (pH 8) solution containing sodium dodecyl sulfate (SDS; 0.6%), EDTA (1 mmol/L), and NaCl (100 mmol/L). DNA (10 μg/sample) from ZenEpo and ZenNeo or ZenEpoK virus-infected cells were digested with restriction enzymes EcoRI or HinIII (GIBCO/BRL), respectively. Digests were size-fractionated in 1% agarose gels and transferred to Hybond C-Extra membranes. The blots were hybridized with the previously described \textsuperscript{32}P-labeled Epo cDNA or Neo cDNA probes. Blots were washed 4 times in 2x saline sodium citrate (SSC)0.1% SDS at room temperature and 3 times in 0.1 x SSC/0.1% SDS at 55°C. Autoradiography was performed using Kodak X-Omat AR film (Kodak Pathé, Marne La Vallée, France).
Phenotypic analysis. Cells were cultured in the presence of Epo or GM-CSF for at least 2 weeks. UT-7 cells were washed in cold phosphate-buffered saline solution (PBS) and incubated (10^6 cells) at 4°C with monoclonal antibodies. After 30 minutes, the cells were washed twice in cold PBS and incubated with a fluorescein-conjugated sheep F(ab)2 antimeublg Ig (Sileneus, Hawthorn, Australia). After 30 minutes of incubation, cells were washed in cold PBS and analyzed with a flow cytometer (FACSort; Becton Dickinson, San Jose, CA). CLB-ery1 (anti-glycophorin A [GPA]) and MY9 (anti-CD33) were purchased from Jansen (Noisy Le Grand, France) and Coulter (Margency, France), respectively. F-2-51 (anti-CD61) was a generous gift from Dr D. Mason (Oxford, UK). Benzidine staining was performed by incubating the cell suspension for 5 minutes in a solution (1:1 [vol/vol]) of benzidine dichlorhydrate (0.2%), acetic acid (0.5 mM), and hydrogen peroxide (0.3%).

Binding studies. Epo was iodinated with a specific activity ranging from 500 to 2,000 Ci/mmol using Iodogen. After an overnight incubation with or without growth factor, cells were washed twice and incubated (5 \times 10^6 cells) for 1 hour at 37°C in 100 µL IMDM containing 10% FCS, 0.1% sodium azide, 25 mM HEPES (pH 7.4), 1×10^6 Epo (~2 \times 10^6 cpm/mL;~1 mMol/L), with or without uniodinated Epo (500 U/mL). Cells were washed 3 times with ice-cold PBS, and cell-bound radioactivity was determined.

Analysis of EpoR phosphorylation. UT-7 cells were cultured in IMDM containing 1% deionized serum albumin (Cohn fraction V; Sigma Chemical Co, St Louis, MO) and iron-saturated human transferrin (100 µg/mL; Sigma) for 18 hours. After an overnight growth factor deprivation in serum-free medium, UT-7 cells were incubated for 10 minutes with or without Epo (10 U/mL), and this stimulation was stopped by the addition of an excess of cold PBS. Cells (10^6 per point) were immediately pelleted and lysed for 30 minutes at 4°C, under constant stirring, in a mild lysis buffer (20 mMol/L Tris [pH 8] solution containing 137 mMol/L NaCl, 2.7 mMol/L KCl, 1% Nonidet P-40, 10% glycerol, 1 mMol/L orthovannadate, 1 mMol/L phenylmethylsulfonyl fluoride, 10 µg/mL leupeptine, 10 µg/mL aprotinin, and 2 µg/mL peptastin). Insolubilized material was removed by centrifugation at 20,000g at 4°C for 20 minutes. Supernatants were preclarified by incubation for 1 hour at 4°C with a nonrelevant rabbit antiserum coupled to protein A beads (Pharmacia) and incubated overnight with either Epo or EpoR antiserum.23 Protein A sepharose beads were added, and immunoprecipitates were solubilized by boiling in SDS lysis buffer containing 1% SDS, 2% EDTA and 5% β-mercaptoethanol and run on a 6% acrylamide gel. After electrotransferred onto nitrocellulose filters, phosphorylated EpoR was detected by immunoblotting with monoclonal anti-phospho-tyrosine antibody (4G10; 2.5 µg/mL; Upstate Biotechnology, Inc, Lake Placid, NY). Immune complexes were revealed using antirabbit IgG antibodies coupled to horseradish peroxidase and by chemiluminescence assay according to the manufacturer’s instructions (Amersham).

RESULTS

Infection and isolation of UT-7 clones. To analyze the effects of an Epo autocrine stimulation, we introduced and expressed the Epo gene in the Epo-responsive UT-7 cell line. To determine whether this stimulation can occur intracellularly, a modified-Epo protein (EpoKDEL) containing a COOH-terminal KDEL ER-retention sequence that prevents its secretion was also expressed. Based on our initial difficulties in transfecting genes in UT-7 cells using electroporation techniques, we used a retroviral infection procedure. Two retroviruses were constructed (Fig 1), one carrying the Epo gene (ZenEpo) and the second carrying the modified EpoKDEL gene (ZenEpoK). Both of them exhibited a neomycin resistance (Neo) gene to be used for selection. To avoid saturation of the retention system, the EpoK gene was expressed, as previously described,2 from a weaker promoter (an internal SV40 early promoter) than the viral LTR. As a control of infection and selection, we analyzed UT-7 cells infected with a Zen Neo virus only carrying the Neo gene (Fig 1).

The selected ZenEpo virus-producing psi-2 clone secreted 20 U of Epo/mL per 2 \times 10^5 cells for every 2 days and infected 20% of FDC-P1 cells. In contrast, the selected ZenEpoK virus-producing psi-2 clone infected a similar percentage of FDC-P1 cells (30%) but only secreted 0.02 U of Epo/mL per 2 \times 10^5 cells for every 2 days, indicating that the retention system was quite efficient.

G418-resistant UT-7 clones, obtained from infection with the supernatant of the bulk G418-resistant psi-CRIP cultures, were individually amplified in liquid cultures. Conditioned media from all the tested G418-resistant UT-7 cells were negative for replication-competent virus. Based on DNA hybridization analysis (Fig 2), we chose several sublines that were independently derived, as shown by each one’s distinct proviral integration site: 5 clones infected with the ZenNeo virus (N1, N4, N6, N10, N15), 2 clones infected with the ZenEpo virus (E1, E5), and 14 clones infected with the ZenEpoK virus (K2, K3, K4, K5, K10, K11, K14, K16, K19, K21, K22, K24, K27, K32).

RNA hybridization analysis (Fig 2) showed transcripts of the expected sizes. Epo mRNA were transcribed from the retroviral LTR via cryptic splicing (approximately 4.0- and 4.5-kb bands). KDEL-modified Epo mRNA were transcribed from the internal SV40 promoter (approximately 1.3-kb band) and the retroviral LTR (approximately 4.0- and 4.5-kb bands). In contrast to the ZenEpo virus-producer psi-2 clone (ΨK22), UT-7 sublines showed a small percentage of EpoK mRNA transcribed from the internal promoter, indicating that the SV40 promoter was less efficient in UT-7 cells than in psi-2 cells (Fig 2). No endogenous Epo transcript (expected size, 1.4 kb) was observed in the ZenNeo virus-infected clones when Northern blot analysis (Fig 2) and reverse transcriptase-PCR procedure (data not shown) were used. Minor mRNA transcripts with unexpected sizes were also observed as an approximately 1.3-kb transcript hybridizing with the Epo probe in ZenEpo virus-infected lines (Fig 2).

Epo production. Epo production from UT-7 clones was measured in cell lysates using RIA and biologic assay. The thresholds of the RIA or the biologic assay for Epo standards in cell lysates were around 0.05 U/mL or 0.1 to 0.2 U/mL, respectively. The high threshold of the biologic assay was caused by some toxicity of the undiluted cell lysates. As shown in Fig 3, Epo was undetectable by RIA in cell lysates from parental and ZenNeo virus-infected UT-7 cells. In contrast, we could detect Epo in all ZenEpo and ZenEpoK virus-infected cells. The Epo concentration detected in the ZenEpo virus-infected clones was higher (5.6 and 3 U of Epo/mL per 20 \times 10^5 cells) than that detected in the ZenEpoK virus-infected clones (from 3 to 0.08 U of Epo/mL per 20 \times 10^5 cells).
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Fig 2. Analysis of proviral location (A) and mRNA expression (B). UT-7 cells were infected with ZenNeo, ZenEpo, or ZenEpoK virus and selected in the presence of G418 to isolate individual clones. DNA and RNA were isolated from each clone and analyzed by gel-blot hybridization. To detect clones with unique integrant, DNA from ZenNeo and ZenEpo virus-infected cells was digested with HindIII. DNA from ZenEpoK virus-infected cells or ZenEpoK virus-producing cells (K22) was digested with EcoRI (see Fig 1). In the right panels, filters were hybridized to a Neo probe, and, in the left panels, samples were hybridized to an Epo probe. Samples include 2 ZenNeo virus-infected clones (N1, N15), 2 ZenEpo virus-infected clones (E1, E5), 2 ZenEpoK virus-infected clones (K4, K10), and the ZenEpoK virus-producing psi-2 cells (K22). Molecular size standards are indicated at left. The 1.5-kb band in the E1 and E5 DNA lanes hybridizing with the Epo probe arises from a HindIII-HindIII fragment of the ZenEpo provirus (Fig 1).

The level of Epo secretion was evaluated by measuring the Epo concentration in conditioned media. RIA (Fig 3) and the biologic assay gave similar results with a threshold close to 0.01 U/mL. E1 and E5 cells secreted around 1.7 and 1 U of Epo/mL per 2 \times 10^9 cells every 2 to 4 days, respectively. In contrast, low amounts of Epo (from 0.02 to 0.04 U/mL per 2 \times 10^9 cells every 2 to 4 days) were detected in the conditioned media from only 3 (K4, K5, K24) of 14 ZenEpoK virus-infected clones. In comparison, the K4 and E5 clones had similar intracellular Epo concentrations, but the K4 cells secreted 97% less Epo (0.03 U/mL) than the E5 cells (1 U/mL). These results show that the ER-retention system efficiently impaired Epo secretion in Epo-producing UT-7 cells and resulted in intracellular accumulation of Epo.

Growth characteristics. Parental UT-7 cell growth is strictly dependent on the presence of growth factors such as Epo or GM-CSF. To know whether retroviral Epo production abolished the growth factor requirement of these cells, virus-infected sublines were cultured without addition of growth factors. No more viable cells were observed in the parental, in the ZenNeo virus-infected cells, and in most of the ZenEpoK virus-infected clones in nonstimulated liquid cultures after 2 weeks. The K4 and K24 lines survived longer in these nonstimulated cultures and showed a variable percentage (around 5% of the total cell number) of viable cells after 1 month of incubation. This percentage sometimes increased with the duration of culture but decreased as soon as the medium was diluted. This survival was not caused by an increased Epo responsiveness, because the amount of Epo resulting in 50% cell survival after 4 days of incubation was similar in the studied ZenNeo (N15) and ZenEpoK (K2, K3, K4) virus-infected clones (31 ± 6 mU/mL) and did not differ when the parental cells were used (33 ± 10 mU/mL). Also,
GM-CSF responsiveness did not appear to be affected by the infection, because the amount of GM-CSF resulting in 50% cell survival after 4 days of incubation was similar in the studied ZenNeo (N1, N15) and ZenEpoK (K2, K3, K4, K10) virus-infected clones (29 ± 10 pg/mL) and did not differ when the parental cells were used (32 ± 12 pg/mL). In contrast to all the other clones, the two ZenEpo virus-infected clones, El and E5, survived and proliferated in unstimulated liquid cultures.

To investigate whether this autonomous growth was caused by either an autocrine or a paracrine stimulation, we analyzed the abilities of the N1, N15, E1, E5, K2, K3, K4, and K10 clones to form colonies in semisolid medium at different cell concentrations in the presence of GM-CSF or without addition of growth factor (Fig 4). In the presence of GM-CSF, the plating efficiency of these clones varied from 10% to 45% according to the experiments. The same variations were observed using the parental UT-7 cells, showing that the infection and the type of virus used did not influence the plating efficiency of these cells. In the absence of growth factor, parental, N1, N15, and K10 lines did not form colonies, whereas the K2 and K3 clones gave rise to rare clusters in some experiments. The only clones that formed reproducible and significant numbers of colonies independent of added growth factor were E1, E5, and K4. The plating efficiency of E1 and E5 clones was not enhanced by the addition of GM-CSF. In contrast, the addition of GM-CSF strongly increased the plating efficiency (by around 80%) and the size of the colonies formed by the K4 clones. Plating efficiency of the K2, K3, K4, E1, and E5 sublines in unstimulated cultures was not dependent on the number of plated cells (from 75 to 2,000 cells/mL), strongly suggesting that colony formation was directly dependent on an autocrine stimulation.

To confirm this hypothesis, the parental, E1, E5, K4, and K3 cells were plated at 1 cell per well in the presence or the absence of GM-CSF. The cloning efficiency of these cultures was always low (around 6% for the parental cells in the presence of GM-CSF), indicating some difficulty in growing this cell line at the unicellular level. Nevertheless, spontaneous growth of isolated cells was observed for the E1, E5, and K4 cells but not for the parental or the K3 cells. For the K4 cells, 1 of 66 isolated cells formed a colony in unstimulated cultures. A larger number of colonies (7 of 69 isolated cells) was obtained in the presence of GM-CSF. These results show that a genuine autocrine stimulation, probably dependent on Epo secretion, was responsible for the autonomous growth and/or survival of the Epo-producing cells.

Effects of Epo neutralizing antibodies. To find out if autocrine stimulation requires secretion, we tested the capacities of anti-Epo antibodies to block this autonomous growth (Fig 5). In all the experiments, the addition of anti-Epo antibodies to unstimulated liquid cultures inhibited both autonomous growth and cell survival of the ZenEpo (E1, E5) and ZenEpoK (K4, K11, K19, K24) virus-infected cells. Such an inhibition was not found in GM-CSF–stimulated cultures, indicating that cell death was not caused by the toxicity of
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Fig 4. Colony formation in semisolid medium from parental UT-7 cells and virus-infected UT-7 clones. From 62 to 1,000 cells were plated per dish (1 mL) in rhGM-CSF-stimulated (●; GM-CSF) or nonstimulated (○; NS) methyl cellulose cultures. Colonies were counted after 7 to 10 days of incubation. Results are mean values (±SD) of triplicate cultures. In nonstimulated cultures, cells had previously been washed twice in IMDM. For the N1, E5, and K10 clones (not shown on this figure), we obtained results similar to those for the N15, E1, and K2 clones, respectively.

The antibodies. The percentage of inhibition was related to the Epo antiserum concentration, and very high concentrations, inhibiting much more than the amount of Epo released in the cell-conditioned media, were required to obtain significant effects on cell survival. For example, an antibody dilution inhibiting approximately 100 U/mL of Epo was necessary to completely block the proliferation and the survival of the E1 cells that secreted only 1 to 2 U of Epo per 2 × 10⁶ cells every 2 to 4 days. These results strongly suggest that the autocrine stimulation induced by Epo in UT-7 cells occurs on the cell surface.

Phenotypes. UT-7 is a pluripotent cell line that undergoes different programs of differentiation depending on the growth factor to which it is exposed. In the presence of Epo, these cells show erythroid features with an increased expression of GPA and hemoglobin (Hb). GM-CSF blocks this Epo-dependent erythroid maturation process. To show whether Epo production influences the differentiation capacities of these cells, we analyzed the expression of erythroid (GPA, Hb), megakaryocytic (CD61), and myeloid (CD33) markers in different infected clones stimulated by GM-CSF and/or Epo. In all analyzed clones, results (Fig 6A and B) showed that GM-CSF blocked the GPA and Hb expression induced by Epo. A large variation in the phenotypes of these clones was observed (Fig 6A), as was the case for clones obtained from the parental cell lines (data not shown). However, no correlation was found, either between Epo production and phenotypes or between Epo production and levels of GPA downregulation by GM-CSF. In addition, the levels of GPA or Hb in the E1 and E5 cells were similar and were
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Fig 5. Effect of Epo-neutralizing antibodies on growth and survival of the virus-infected clones. Microwell assays were performed by adding 10 μL volume of IMDM, 10% FCS, containing 200 GM-CSF-stimulated (2.5 ng/mL; *) or nonstimulated (NS; 0) calls to 5 μL volume of serial twofold dilutions of Epo antibodies. Viable cells were counted between 7 and 10 days of incubation at 37°C. Results are mean values (± SD) of duplicated culture. In the same experiment, 200 nonstimulated cells were added to IMDM, 10% FCS (0) to assay the autonomous growth (greater than 200 viable cells) or the survival (less than 200 viable cells) of the cells. Results are mean values (± SD) of at least 12 culture wells. No viable UT-7 parental cell were ever observed after 5-day incubations in nonstimulated culture wells. For the E5, K11, and K24, results of one typical experiment are shown. For the E1 cells, mean values (± SD) of three experiments, using high concentrations of antibodies, are shown. An Epo antibody (serum or purified IgGs) dilution of 1/1,000 inhibited around 1 U/mL of rhEpo.

lowered by GM-CSF in both unstimulated (data not shown) and Epo-stimulated cultures. These data indicate that GM-CSF downregulates the erythroid differentiation induced in UT-7 cells either by Epo autocrine stimulation or by exogenously added Epo. Therefore, retroviral Epo production had no differentiative activities when retained in the ER and did not modify the differentiative capacities of this cell line.

EpoR features. We next examined whether Epo production influences EpoR expression and activation. The UT-7 cell line shows around 7,000 EpoR per cell, which are downmodulated by GM-CSF and Epo at the mRNA level. Binding data showed a reduction in the number of 125I-Epo binding sites for all analyzed clones in comparison with the parental UT-7 cells (Fig 7A). ZenNeo and ZenEpoK virus-infected clones showed a similar reduction in the number of binding sites, the mean values ± SD being 55% ± 17% and 50% ± 11%, respectively. In contrast, ZenEpo virus-infected clones expressed a more drastic reduction in Epo binding sites than the other clones (mean value ± SD, 19% ± 7%). This reduction was similar to the one (78%) observed with the parental UT-7 cell line stimulated overnight by Epo. This cannot be explained by EpoR occupancy, because a 2-hour incubation of the parental cells with Epo only slightly reduced the number of binding sites (8%; data not shown), as was found in previous studies with normal cells. RNA hybridization analysis (Fig 7B) showed low levels of the EpoR mRNA in the Epo-stimulated or GM-CSF-stimulated parental UT-7 cells and in the two starved ZenEpo virus-infected clones, compared with the levels found in starved ZenEpoK virus-infected clones and controls. These results show that Epo autocrine stimulation downmodulates the EpoR, at the mRNA level, similarly to the way the addition of Epo does in the parental line. This process was not observed in KDEL-modified Epo-producing clones which
strongly suggests that this Epo downmodulation is totally dependent on Epo secretion.

Phosphorylation of the EpoR is one of the earliest events occurring after Epo stimulation. This phosphorylation is a rapid and transient event. Its signal, detectable only in previously starved cells, is at its maximum after 1 to 2 minutes of Epo stimulation and becomes undetectable 30 minutes after this stimulation. In the continuous presence of Epo, owing to the permanent phosphorylation-dephosphorylation process, the phosphorylation of the EpoR is only barely de-
Fig 7. EpoR expression from the parental and infected lines. (A) Specific binding of $^{125}$I-Epo to parental (■), ZenNeo (□), ZenEpo (□) or ZenEpoK (□) virus-infected UT-7 cells. Results are expressed in percentages of binding compared with that of the starved parental UT-7 cells. Binding experiments were performed in triplicate. In brackets is the number of experiments. Results are mean values (± SD) of the different experiments (—) or the single experiment (—). All virus-infected cells were analyzed after an overnight growth factor starvation. Control parental cells were analyzed after an overnight growth factor starvation, an overnight GM-CSF (2.5 ng/mL) stimulation, or an overnight Epo (2 U/mL) stimulation. (B) Northern blot analysis of the EpoR mRNA from Epo or GM-CSF-stimulated parental line and starved parental and infected lines. Ethidium bromide staining of the ribosomal RNA are indicated as a control of the amount of loaded RNA from each sample.

**DISCUSSION**

This report describes an in vitro model of Epo autocrine stimulation. We have used the pluripotent human UT-7 cell line whose growth, differentiation, and survival are strictly dependent on the addition of growth factors including Epo and GM-CSF. We show that retroviral transfer of the Epo cDNA abolished the growth factor requirement of these cells. Spontaneous growth was not cell density-dependent and occurred at unicellular level, thus showing a genuine autocrine mode of stimulation. This autocrine stimulation seemed to be mediated through Epo secretion, because the addition of Epo-neutralizing antibodies inhibited the autonomous growth. In an attempt to confirm this result, Epo secretion was blocked using the KDEL retention signal. This approach allowed an intracellular Epo accumulation with little or no Epo secretion. Our data show that a complete Epo secretion blockage resulted in the absence of autonomous growth, whereas a leaky retention system resulted in more in cell survival than in a true long-term proliferation. Furthermore, in contrast to Epo expression, KDEL-modified Epo expression did not induce EpoR downregulation or phosphorylation. All these data strongly suggest that localization of Epo in the ER cannot induce an autocrine stimulation in the UT-7 cell line. Such stimulation is dependent on Epo secretion and appears to take place entirely on the cell membrane.

The C-terminal tetrapeptide KDEL signal is recognized by a membrane-bound receptor, which is responsible for the retrieval of ER proteins from the Golgi apparatus. Studies by Pelham and colleagues indicate that the KDEL-bearing molecules (1) are delivered into the ER, (2) escape from the ER, (3) bind to their receptor in the Golgi apparatus or in an intermediate compartment between it and the ER, and (4) are retrieved by these receptors, which cycles them back to the ER, where (5) they are finally released until their next exit from the ER. This recycling pathway may allow KDEL-modified ligands (unbound from the membrane-anchored KDEL receptor) to interact freely with their physiologic receptor within the ER and, possibly, within the Golgi apparatus. Indeed, as examples of such possible interactions, the expression of IL-6 or soluble CD4 protein modified with a COOH-terminal KDEL sequence have been shown to modify the metabolism of the IL-6 receptor or the gp120, respectively. As expected, the transfer of the Epo gene modified with this signal led to the impairment of Epo secretion in
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Fig 8. EpoR tyrosine phosphorylation from Epo-stimulated and unstimulated parental UT-7 cells and UT-7 virus-infected clones. UT-7 cells were incubated for 10 minutes in the presence (+) or absence (−) of Epo (10 U/mL) and solubilized under mild lysis conditions. Lysates were immunoprecipitated with anti-EpoR antibodies and analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. Phosphorylated proteins were detected by immunoblotting with antiphosphotyrosine antibodies. Cells used were uninfected UT-7 cells (lanes 1 and 2), ZenNeo virus-infected cells (N1, lanes 3 and 4), ZenEpo virus-infected cells (E5, lanes 5 and 6), ZenEpoK virus-infected cells (K4, lanes 7 and 8; and K10, lanes 9 and 10). Size markers are indicated on the right (× 10^3).

14 independent clones. A leaky retention system, which was probably because of the saturation of this receptor-mediated process, was observed in only 3 clones. A total retention of Epo inside the cell led to the failure of autonomous growth. This failure was not caused by modifications of Epo biologic activity, because immunoreactive KDEL-modified Epo, present in cell lysates or conditioned medium, had the same biologic activity as unmodified Epo, thus demonstrating that the KDEL modification did not modify the affinity of Epo for its receptor. Another explanation could be that intracellular Epo concentration in these clones, probably reduced through the use of an internal promoter, was too low to induce an autocrine stimulation. However, intracellular Epo concentrations in Epo KDEL clones were estimated to be at least from 5 to 200 times more elevated than those required to stimulate the parental cell line (approximately 200 mU/mL of Epo). Moreover, two sublines E5 and K4, obtained with the Zen Epo and Zen Epo KDEL virus, respectively, showed identical intracellular Epo concentrations but showed distinct growth behaviors in nonstimulated cultures. The E5 subline, which secreted large amounts of Epo, showed a complete autonomous growth, whereas the K4 subline, which secreted low amounts of Epo, only survived longer than the parental cells and showed a minimal proliferation or cell survival in nonstimulated cultures. Interestingly, the Epo concentrations released by the clones with a leaky retention system (approximately 30 mU/mL) were close to those inducing a similar partial inhibition of apoptosis in the parental cell line, ie, approximately 50% survival. In contrast, regardless of their intracellular Epo concentration, the Epo KDEL clones showing a total Epo retention in the ER did not survive or proliferate for long periods in the absence of exogenous growth factor. All these results strongly suggest that the growth and survival of the Epo KDEL clones, as well as the Epo clones, were strictly dependent on Epo concentrations released by the cells in the medium. In conformity with these results, when secreted Epo was neutralized by Epo antiserum in Epo or Epo KDEL clone cultures, autonomous survival or proliferation were almost totally inhibited, depending on antibody concentration. The incomplete effect of this antibody inhibition may have resulted either from similar binding affinities for Epo of the antibodies and the receptor or from antibody consumption in the vicinity of the membrane where Epo and EpoR are continuously produced.

Because cell survival and proliferation are only two of the three processes mediated by Epo, we investigated whether intracellular Epo stimulation could eventually mediate an erythroid differentiation. As was recently shown for the G-CSF receptor, the differentiative signal pathway induced by the EpoR may be different from the proliferative one. Indeed, it has been reported that herbimycin treatment inhibits the proliferative activity of Epo without affecting its differentiative properties. However, despite the large number of isolated clones, we were unable to find any differences in the phenotype of the Epo-producing clones in comparison with the control Neo clones in GM-CSF-stimulated cultures. As in the parental cell line and the control Neo clones, exogenous Epo induced an erythroid differentiation process that was blocked...
by the addition of GM-CSF. The only difference was observed in Epo-secreting clones where the addition of exogenous Epo did not modify the phenotype. However, the addition of GM-CSF still reverted the erythroid phenotype of these clones. Thus, Epo production did not change the differentiation capacities of this cell line and had no erythroid differentiation activity when Epo was retained in the ER.

From all these data, it appears that intracellular Epo was not effective in the induction of cell survival, proliferation, or differentiation. We wonder whether this was because of an inability of Epo to stimulate its receptor inside the cell. In many models, intracellular autocrine stimulation modifies receptor metabolism, inducing its downregulation and eventually its activation. However, this intracellular activation of the receptor is not always sufficient and may also require expression on the cell surface for complete transformation or cell proliferation. EpoR belongs to the superfamily of cytokine receptors. Tyrosine phosphorylations are essential for its signal transduction, although these receptors have no intrinsic kinase activity. Several studies have recently shown that these receptors are closely associated with cytoplasmic kinases. In the parental cell line, we showed that stimulation by Epo induces a downregulation of the EpoR (binding and mRNA) and the phosphorylation of several proteins including the EpoR. To study the metabolism of EpoR induced by an Epo autocrine stimulation, we compared Epo binding sites and EpoR phosphorylation of the ZenNeo, Zen-Epo, and ZenEpoK virus-infected clones. KDEL Epo and Neo expressing cells showed a similar number of surface EpoR. Surprisingly, all these clones showed a lower number of EpoR than the parental cell line. The most likely explanations were that either retroviral infection or selection procedures are responsible for this lower level of surface EpoR. Nevertheless, the Epo clones showed a further twofold reduction in their Epo-binding capacities compared with those of the control ZenNeo virus-infected clones. A similar reduction was also observed at the EpoR mRNA levels. Thus, in contrast to Epo, the KDEL-modified Epo production did not induce any downregulation of the EpoR. Phosphorylation of the EpoR in response to exogenous Epo was shown in the Neo control clone as well as in the clones expressing KDEL-Epo but not in the one expressing Epo. These data indicate that the EpoR is constitutively activated in the Epo clones but not in the KDEL-Epo clones. Two main hypotheses could explain the absence of EpoR activation in the Epo KDEL clones. First, to be active, EpoR must be coupled to a kinase, and recent evidence suggests that this kinase is JAK2. If this association does not take place in the ER, the binding of Epo to its receptor in the ER will be unable to transduce a message. Second, no Epo-EpoR complexes can be formed in the ER. The absence of EpoR downregulation in Epo KDEL clones, which is observed for some other receptors when coexpressed with their KDEL-modified ligands, provides strong evidence in favor of this second hypothesis. One can argue that the KDEL modification has impaired the Epo-EpoR interaction, but this is rather unlikely, because EpoKDEL has the same biologic activity as Epo. Furthermore, our preliminary experiments have failed to detect any intracellular immunocomplexes not only between EpoR and Epo KDEL, but also between EpoR and unmodified Epo. The most likely explanations for the absence of intracellular Epo-EpoR complexes might be (1) that both molecules traffic in two different ER compartments or (2) that the structure of the EpoR in the ER does not allow Epo binding. In the Friend model, intracellular interaction of EpoR and gp55 actually occurs in the ER inducing intracellular gp55-EpoR complexes and EpoR downregulation. This difference may be explained by the fact that Epo and gp55 have different binding sites on the EpoR. However, elegant data show that EpoR/gp55 complexes are also detectable at the cell surface and that growth stimulation is indeed related to this low number of EpoR/gp55 complexes present on the cell surface. Therefore, even in this model, only the external activation seems crucial.

The KDEL retention signal has previously been used with other factors to locate the cellular sites of autocrine mechanisms, and different results have been obtained, depending on the growth factor studied. Intracellular retention of IL-3 or the v-sis product did not impair their proliferative activities. In contrast, the autocrine activity of K-FGF was impaired after addition of the KDEL signal. These differences may be related to different mitogenic pathways or receptor-ligand binding requirements but do not seem to be dependent on their receptor family. K-FGF and v-sis receptors both have intrinsic tyrosine kinase activity, and the IL-3 receptor belongs to the same receptor family as the EpoR. An alternative explanation is that, in many previous studies, the existence of a leaky retention system has not been extensively examined. Recent experiments using the v-sis or IL-3 genes engineered for intracellular retention suggest that induction of proliferation was also related to secretion.

In conclusion, Epo autocrine stimulation in UT-7 cells was shown to be strictly dependent on Epo secretion and resulted in biologic effects similar to exogenously added Epo. These results strongly suggest that autocrine stimulation differs from endocrine or paracrine stimulation only by the absence of physiologically regulated growth factor production.

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Autocrine stimulation by erythropoietin (Epo) requires Epo secretion

JL Villeval, MT Mitjavila, I Dusant-Fourt, F Wendling, P Mayeux and W Vainchenker