

Erythropoietin induces cancer cell resistance to ionizing radiation and to cisplatin

Alexandre I. Belenkov,¹ George Shenouda,¹ Ekatarina Rizhevskaya,¹ Denis Cournoyer,² Jean-Philippe Belzile,² Luis Souhami,¹ Slobodan Devic,³ and Terry Y.K. Chow^{1,4}

¹Department of Oncology, Division of Radiation Oncology, Montreal General Hospital; ²Department of Human Genetics, Montreal General Hospital and Research Institute; and ³Department of Medical Physics, McGill University; ⁴Oncozyme Pharma, Inc., Montreal, Quebec, Canada

Abstract

Recent studies suggest that erythropoietin plays an important role in the process of neoplastic transformation and malignant phenotype progression observed in malignancy. To study the role of erythropoietin and its receptor (EPOR) on the response of cancer cells *in vitro*, we used two solid tumor cell lines, namely the human malignant glioma cell line U87 and the primary cervical cancer cell line HT100. All experiments were done with heat-inactivated fetal bovine serum in order to inactivate any endogenous bovine erythropoietin. The expression of the EPOR in these cells was confirmed with immunoblot techniques. The addition of exogenous recombinant human erythropoietin (rhEPO) induces the cancer cells to become more resistant to ionizing radiation and to cisplatin. Furthermore, this rhEPO-induced resistance to ionizing radiation and to cisplatin was reversed by the addition of tyrphostin (AG490), an inhibitor of JAK2. Our findings indicate that rhEPO result in a significant, JAK2-dependent, *in vitro* resistance to ionizing radiation and to cisplatin in the human cancer cells lines studied in this report. [Mol Cancer Ther 2004;3(12):1525–32]

Introduction

Recombinant human erythropoietin (rhEPO) has been widely used in the clinic for treatment of anemia associated with cancer, with the potential for a possible concomitant effect to increase tumor oxygenation and thereby, radio-

curability (1). Hypoxic cells are more resistant to inactivation by low-linear energy transfer X- and γ -rays than well-oxygenated cells (2, 3). Consequently, the radiosensitivity of malignant neoplasms is decreased by the presence of either chronically or transiently hypoxic neoplastic cells. Moreover, hypoxic neoplastic cells are clinically more aggressive and have a more metastatic potential than their well-oxygenated counterparts (4, 5).

Recently, experimental findings from various laboratories showed that the expression of erythropoietin and its receptor (EPOR) is not restricted to the hematopoietic system. Erythropoietin and EPOR are expressed in cancer cells from the kidney (6), brain (7), breast (8), and as well as other tissues (9–12). These findings suggest that the biological effects of erythropoietin are not limited to the regulation of erythropoiesis. Erythropoietin may have a broader role in regulating normal and neoplastic cell survival and in enhancing the process of angiogenesis. An autocrine erythropoietin signaling has been recently showed by Acs et al. (13) to inhibit hypoxia-induced apoptosis in human breast carcinoma cells.

Erythropoietin is a $M_r \sim 30,000$ glycoprotein hormone produced by kidney and fetal liver. It acts via the EPOR to stimulate growth, prevent apoptosis, and induce differentiation of RBC precursors (14, 15). The interaction between erythropoietin and its receptors is in the ratio of one molecule of erythropoietin to two molecules of EPOR resulting in a triggering receptor-mediated signaling event with a subsequent receptor internalization and degradation (16). Erythropoietin receptor, although a member of the cytokine receptor family, has no intrinsic kinase activity. It binds and activates intracellular tyrosine kinases [Janus kinase (JAK)] to elicit its mitogenic effects (17). JAKs represent a family of cytoplasmic tyrosine kinases that play a major role in cellular signaling process (18, 19). Having bound to a ligand, JAKs are activated and subsequently phosphorylate a number of substrates, including the receptors themselves. The phosphorylated receptors provide docking sites for the SH2 domain containing the signal transducers and activators of transcription (STAT) transcription factor family. Subsequently, JAKs phosphorylate the STATs on a single tyrosine residue, causing STATs dimerization, migration to the nucleus, and regulation of gene transcription. Among different JAKs, the JAK2 is of particularly interest. It is activated upon binding of erythropoietin to its receptor (20). A carboxyl-truncated JAK2 mutation caused significant negative effects on erythropoietin-induced JAK2 activation and mitogenesis (21). JAK2 activation subsequently leads to the phosphorylation and activation of STAT5 (22). In addition to JAK2/STAT5, erythropoietin also activates other signal transduction pathways, such as the extracellular signal-regulated kinase pathway and signal transduction molecules, such as

Received 5/11/04; revised 8/25/04; accepted 10/15/04.

Grant support: National Cancer Institute of Canada and Canadian Cancer Society.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Terry Y.K. Chow, Department of Oncology, Division of Radiation Oncology, Montreal General Hospital, 1650 Cedar Avenue, Montreal, Quebec, Canada H3G 1A4. Phone: 514-934-1934 ext. 42904; Fax: 514-934-8202. E-mail: tchow@po-box.mcgill.ca
Copyright © 2004 American Association for Cancer Research.

SHC/GRB-2, CRK-1, Vav, phosphatidylinositol 3-kinase, and the protein phosphatases SHP-1 and SHP-2 (23–27). Activation of these pathways requires an intact form of the EPOR.

In this study, we investigated the *in vitro* effect of erythropoietin/EPOR-mediated JAK2/STAT5 signaling pathway in cancer cells exposed to ionizing radiation and to cisplatin.

Materials and Methods

Cell Lines and Culture

U87 and K562 cells were obtained from American Type Culture Collection (Rockville, MD). HT100 primary cells and cervical carcinoma biopsy samples designated HT67, HT219, HT270, and HT280 were generously provided by Dr. Joan Turner (Cross Cancer Institute, University of Alberta). U87 and HT100 cells were cultured in DMEM and K562 cells were cultured in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% (v/v) of fetal bovine serum (FBS), batch number 22524017 (Bio-Media Canada, Drummondville, Quebec, Canada). FBS heat inactivation was conducted at 56°C for 30 minutes. Heat inactivation was done in order to inactivate any endogenous erythropoietin in FBS before its use in the experiments. rhEPO, EPREX epoetin alfa, was obtained from Ortho Biotech (Janssen-Ortho, Inc., Toronto, Ontario, Canada). Tyrphostin AG490 was obtained from Sigma Chemical Co. (St. Louis, MO). All cell lines were maintained in a humidified atmosphere of 5% CO₂ at 37°C.

Preparation of Whole-Cell Extracts

Whole-cell extracts were prepared by a single freeze-thaw cycle (28). Briefly, cells were harvested, washed with ice-cold PBS and lysis-supporting buffer [25 mmol/L KCl, 10 mmol/L NaCl, 1 mmol/L MgCl₂, 10 mmol/L HEPES-KOH (pH 7.2), 0.1 mmol/L EDTA, 2 µg/mL leupeptin (Sigma Chemical), 2 µg/mL aprotinin (Sigma Chemical), 0.5 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride], pelleted, and resuspended in a lysis-supporting buffer to a final volume of 2.5 times the original packed cell volume. Resuspended cells were incubated on ice for 10 minutes, frozen by plunging into liquid nitrogen, quickly placed at 37°C until slightly thawed, and placed on ice. NaCl and MgCl₂ concentrations of fully thawed samples were adjusted to 0.5 and 10 mmol/L, respectively, by adding high-salt buffer (5 mol/L NaCl, 100 mmol/L MgCl₂, and 5 mmol/L DTT). Cell extracts were incubated on ice for 5 minutes and microcentrifuged at 10,000 × g for 5 minutes at 4°C. Supernatants were collected, and protein concentrations were quantified using the method of Bradford (29).

Immunoblotting

Cell extracts containing 10 to 50 µg of protein were separated by SDS-PAGE and transferred by electrophoresis onto nitrocellulose membranes (Millipore, Bedford, MA) for 1 hour in 25 mmol/L Tris-HCl, 192 mmol/L glycine, and 20% methanol. Membranes were blocked in TBS (Tris-HCl) containing 5% nonfat milk and probed with anti-EPOR

(C-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and α-tubulin monoclonal antibodies (NeoMarkers, Fremont, CA). Antigen/antibody complexes were detected by horseradish peroxidase-conjugated anti-mouse antibody and enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ), according to the manufacturer's instructions. The bands were quantified by densitometric analysis using Scion Image software (Scion Co., Frederick, MD).

Immunocytochemistry of U87 and HT100 Cells

U87 and HT100 cells were cultured for 24 hours on eight-well Lab-Tek II Chamber Slides (Nalge Nunc International Co., Naperville, IL). Cells were washed gently with PBS and fixed for 3 minutes with ice-cold 100% acetone. The cells were covered with 5% bovine serum albumin in PBS and incubated for 1 hour at room temperature. The cells were washed once with PBS and incubated with mouse monoclonal anti-human EPOR fluorescein-conjugated antibody (R&D Systems, Minneapolis, MN) overnight at 4°C, at 1:200 dilution in 1% bovine serum albumin in PBS. Following incubation, the cells were washed twice with PBS for 5 minutes and coverslips were mounted using a nail polish and examined by fluorescence microscope.

Analysis of Erythroid Cell Differentiation

The *in vitro* biological activity of rhEPO was determined by measuring the hemoglobin production of the human erythroleukemia cell line K562. Fetal bovine serum (FBS) heat inactivation was done in order to inactivate any endogenous erythropoietin in FBS before its use in the experiments. Heat inactivation of FBS was a critical and necessary step in the experimental design of this study, in order to minimize any confounding effect of endogenous erythropoietin in FBS that may otherwise mask the effect of exogenous rhEPO when the latter is added. K562 cell suspension was diluted in culture media supplemented with heat-inactivated or regular FBS at 2.5%, 5.0%, 10.0%, and 20.0% (v/v) in the presence or absence of 0, 2.5, 5.0, and 10.0 units/mL of rhEPO to yield 1.0 × 10⁵ cells/mL. The cell suspension (100 L) was transferred into a 96-well microtiter plate using a multichannel pipette. Following 96 hours of incubation, the cells were assayed for the presence of hemoglobin using the 2,7-diaminofluorene colorimetric reagent as previously described (30). Briefly, following 96 hours of incubation, the cells were centrifuged and the growth medium aspirated. The cells were lysed with ice-cold water containing 1% of NP40, and cell lysates were adjusted to protein concentration of 2.5 mg/mL in 0.2 mL volumes. Working 2,7-diaminofluorene solution (0.6 mL) was added to the samples and the mixture was incubated for 5 minutes at room temperature. Absorbance measurements were made at 610 nm within 10 minutes following the incubation period.

U87 and HT100 Cell Sensitivity to Ionizing Radiation and to Cisplatin

The effect of ionizing radiation or cisplatin (Sigma Chemical) treatment on the survival of U87 and HT100 cells cultured in 10% heat-inactivated FBS and stimulated with 30 units/mL of rhEPO, was measured using the

3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and clonogenic survival assays. Tyrphostin B42 (AG490, Calbiochem, La Jolla, CA) was used as a specific inhibitor of JAK2 tyrosine kinase activity. For the MTT assay, the cells were plated on 96-well plates at 5,000 cells/well. The cells were allowed to attach to the bottom of the wells for 6 hours after which the cells were exposed to various concentrations of cisplatin. Following 6 hours of incubation, the cisplatin-containing medium was aspirated. The cells were washed gently with 0.2 mL of serum-free medium and incubated with 0.2 mL of corresponding growth media for 72 hours. A solution of MTT (50 μ L of a 5 mg/mL solution in PBS) was then added, and the cells were incubated for another 3 hours. The medium was removed and replaced by 100 μ L of DMSO, and the plate absorbance was measured at 570 nm. For the clonogenic survival assay the cells were plated onto 25-cm² tissue culture flasks (Sarstedt, Newton, NC) at various concentrations (200–15,000 cells/well) and exposed to γ -irradiation from a ¹³⁷Cs source for time periods equivalent to 2.0, 4.0, 6.0, 8.0, and 10.0 Gy. After 14 days, cells were washed with PBS, fixed with gluteraldehyde, and stained with 1% crystal violet. Washed dishes were dried, and colonies containing >50 cells were scored. The surviving fraction was plotted on a log scale against drug concentration or radiation dose.

Statistical Analysis

The two-tailed Student's *t* test was used to compare statistical significance among various groups.

Results

Expression of EPOR in U87 and HT100 Cells

Western blot analysis of U87, HT100 cells, and cervical carcinoma biopsy samples designated HT67, HT219, HT270, and HT280 showed that a specific immunoreactive band, of $M_r \sim 66,000$ and corresponding to the EPOR protein, was present in all samples (Fig. 1A). Immunocytochemical labeling showed that both U87 and HT100 cells expressed EPOR on the cell surface (Fig. 1B).

Analysis of Erythroid Cell Differentiation

Our initial results of U87 and HT100 cell proliferation and survival showed large variations between experiments done with different batches of FBS. We also noticed that addition of the rhEPO to the growth medium had little or no effect when the FBS used for preparation of the growth medium was not heat inactivated. It has been previously reported that heat inactivation of serum samples at 56°C destroyed erythropoietin in specimens tested by the mouse spleen cell assay (31). In the light of our initial results, we hypothesized that the effect of rhEPO on U87 and HT100 cell proliferation and survival might be masked by the levels of endogenous erythropoietin present in normal noninactivated FBS, and that this endogenous level of bovine erythropoietin varies from one batch to another. It has been previously reported that rhEPO induced differentiation in the K562 human erythroleukemia cell (32–34). Therefore, we used this method to test our

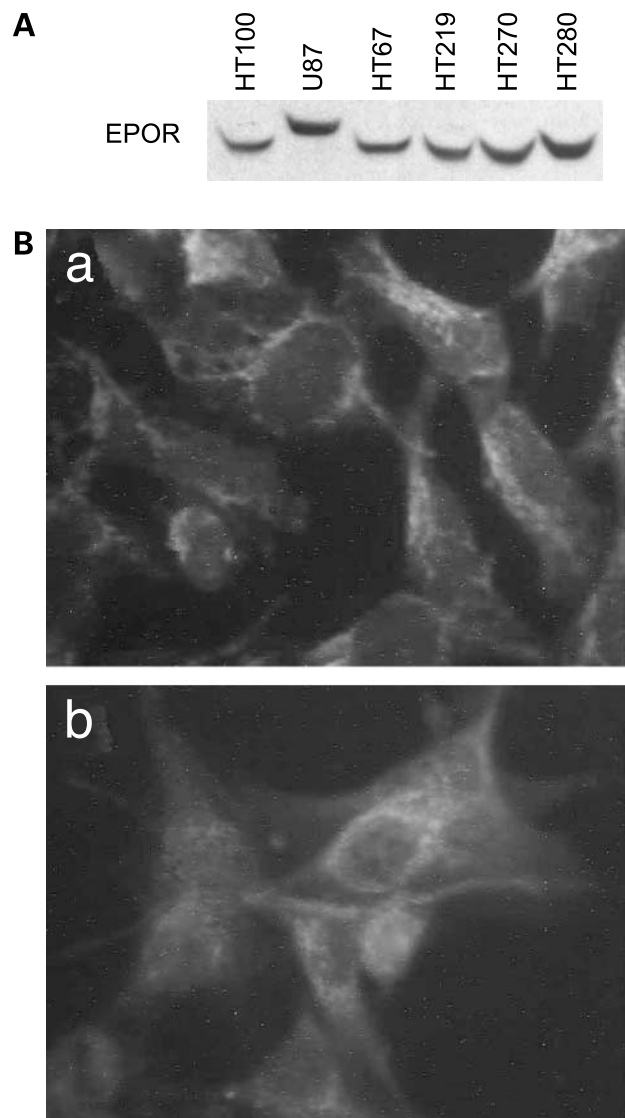


Figure 1. **A**, expression of EPOR in U87, HT100 cells, and cervical carcinoma biopsy samples designated HT67, HT219, HT270, and HT280. Western blotting demonstrated that a specific immunoreactive band corresponding to $M_r \sim 66,000$ to the EPOR protein was present in all cell lines and biopsy samples studied. **B**, EPOR immunocytochemistry. U87 (**a**) and HT100 (**b**) cells were cultured for 24 hours on eight-well Lab-Tek II Chamber Slides. The cells were then fixed and subjected to immunofluorescence analysis with anti-human EPOR fluorescein-conjugated antibody. Note the uniform labeling of EPOR in both cell lines. Representative of four experiments.

hypothesis by studying the effect of FBS heat inactivation and the effect of rhEPO addition in K562 human erythroleukemia cells. Erythroid differentiation of K562 cells was induced by culturing the cells for 4 days in medium containing heat-inactivated or normal noninactivated FBS at 2.5%, 5.0%, 10.0%, and 20.0% (v/v) and in the presence or absence of 0, 2.5, 5.0, and 10.0 units/mL of rhEPO (Fig. 2A and B). Addition of rhEPO to heat-inactivated serum led to a significant increase in K562

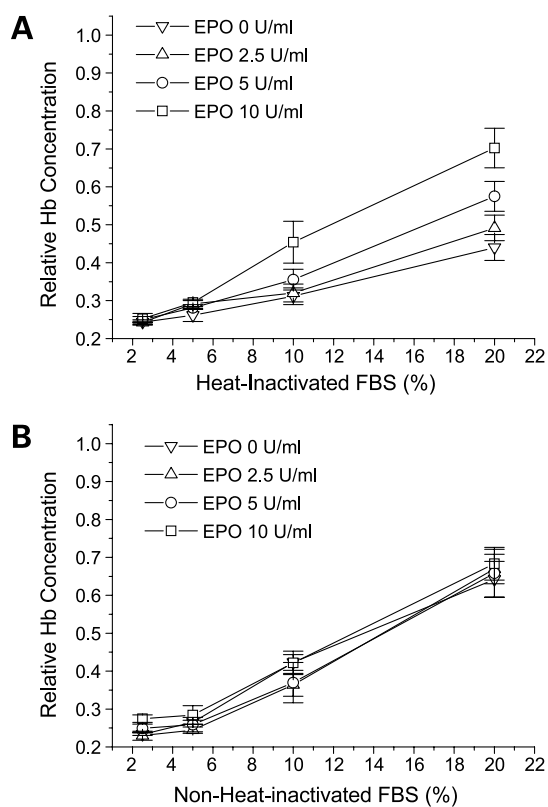


Figure 2. Analysis of erythroid cell differentiation. K562 cell suspension was diluted in culture media supplemented with (A) heat-inactivated and (B) nonheat-inactivated FBS in the presence of 0, 2.5, 5.0, and 10.0 units/ml of rhEPO to give the initial cell number of 1.0×10^5 cells/mL and cultured for 4 days. Addition of rhEPO to heat-inactivated serum led to a significant increase in K562 differentiation in a dose-dependent manner (A), whereas no effect of rhEPO addition was seen in the samples where nonheat-inactivated FBS was used to prepare growth medium (B).

differentiation in a dose-dependent manner (Fig. 3A), whereas no effect of rhEPO addition was seen in the samples where normal noninactivated FBS was used to prepare growth medium (Fig. 2B).

In vitro Sensitivity of U87 and HT100 Cells to Ionizing Radiation and Cisplatin

Colony survival and the MTT assays were used to determine the effect of rhEPO on the sensitivity of U87 and HT100 cells to ionizing radiation and to cisplatin, respectively. Both cell lines were cultured in growth medium containing heat-inactivated FBS. At first, we determined if rhEPO addition alone would affect cell sensitivity to ionizing radiation and cisplatin. Treatment of U87 and HT100 cells with 30 units/mL of rhEPO was associated with a decrease in radiation sensitivity compared to untreated controls (ID_{50} : 7.4 versus 10.1 Gy and 4.1 versus 5.0 Gy, respectively; $P < 0.01$; paired t test) (Fig. 3; Table 1). Similarly, treatment of U87 and HT100 cells with 30 units/mL of rhEPO was associated with decrease in sensitivity to cisplatin in both cell lines (ID_{50} : 14.0 versus 28.6 mol/L and 6.7 versus 15.7 mol/L, respectively;

$P < 0.01$; paired t test; Fig. 4; Table 1). To investigate whether inhibition of JAK2 kinase, an effector of the EPOR, affects U87 and HT100 cell sensitivity to ionizing radiation and to cisplatin, we tested the effect of a specific JAK2 inhibitor tyrphostin (AG490) on the erythropoietin-induced cell survival. As illustrated in Figs. 3 and 4 and Table 1, the administration of AG490 alone did not affect U87 and HT100 cell sensitivity to ionizing radiation or to cisplatin, respectively (ID_{50} : 7.4 versus 6.9 Gy and 4.1 versus 4.0 Gy, respectively; $P < 0.01$; paired t test). However, when AG490 was present in the culture medium of cells treated with rhEPO, the rhEPO-induced cell survival of U87 and HT100 cells was abolished and the level of sensitivity returned back to control values. The result suggested that the observed effect of rhEPO-induced resistance to ionizing radiation and cisplatin was JAK2 dependent.

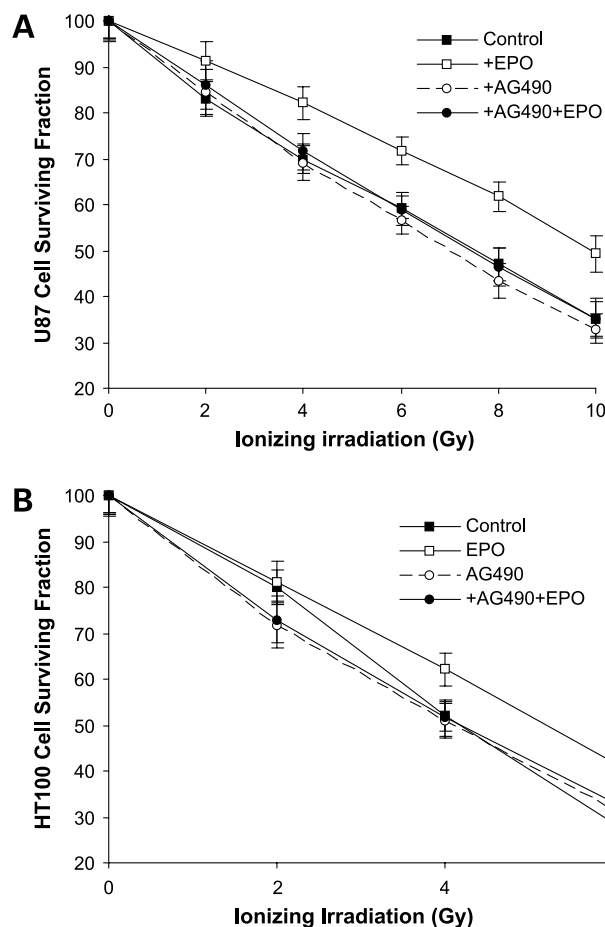


Figure 3. Survival of U87 and H100 cells as measured by colony formation after exposure to various doses of γ -irradiation. (A) Comparison of γ -irradiation sensitivity between untreated (Control), EPO-treated, tyrphostin-treated (AG490), and EPO + AG490-treated U87 cells. (B) Comparison of γ -irradiation sensitivity between untreated (Control), EPO-treated, tyrphostin-treated (AG490), and EPO + AG490-treated HT100 cells. Points, means from three independent experiments; bars, \pm SE.

Table 1. Sensitivity of U87 and HT100 cells treated with rhEPO, AG490, and rhEPO + AG490 to ionizing radiation and cisplatin

Cell Line Treatment	Control*	rhEPO (30 units/mL)*	AG490 ($\mu\text{mol/L}$)*	rhEPO + AG490*
U87, ionizing radiation (Gy)	7.4 (± 0.38)	10.1 (± 0.45)	6.9 (± 0.43)	7.3 (± 0.41)
U87, cisplatin ($\mu\text{mol/L}$)	14.0 (± 0.67)	28.6 (± 0.58)	12.1 (± 0.41)	13.8 (± 0.51)
HT100, ionizing radiation (Gy)	4.1 (± 0.27)	5.0 (± 0.25)	4.0 (± 0.36)	4.1 (± 0.32)
HT100, cisplatin ($\mu\text{mol/L}$)	6.7 (± 0.31)	15.7 (± 0.39)	6.4 (± 0.28)	7.0 (± 0.37)

NOTE: Values represent the radiation dose or drug concentration that produces 50% cytotoxicity. All values are the means of three independent experiments (\pm SE).

* $P < 0.01$ with 7.4 versus 10.1 Gy and 4.1 versus 5.0 Gy of radiation, respectively. Likewise, $P < 0.01$ with 14.0 versus 28.6 mol/L and 6.7 versus 15.7 mol/L of cisplatin, respectively.

Discussion

Anemia was shown to be a strong predictor of poor outcome for patients treated with radiotherapy (35). Attempts have been made in order to increase oxygen delivery to tumors and to improve treatment response and overall survival. One method that may improve oxygen delivery to hypoxic tumor tissues is increasing the hemoglobin levels. Indeed, patients with cervical carcinoma who received blood transfusions showed improved outcomes with radiation therapy (36). However, due to the risk of HIV and hepatitis transmission from contaminated blood, the patients are often not transfused unless their hemoglobin levels fall below 100 g/L. An alternative method for maintaining higher red cell count is the administration of rhEPO. Several studies have shown that rhEPO was effective in increasing hemoglobin levels in cancer patients and that the increase was also associated with an improvement in patient-reported quality of life (37). Whereas there is evidence of an erythropoietin enhancement of radiosensitivity of xenografted human tumor in anemic nude mice, the radiosensitivity of tumor in nonanemic animals' tumor could not fully be restored (38). However, recent experimental evidences from several laboratories (10–12) have raised the possibility of adverse effects related to rhEPO treatment in cancer patients (39, 40). Western blot analysis of U87, HT100, and cervical carcinoma biopsy samples designated HT67, HT219, HT270, and HT280 showed that a specific immunoreactive band corresponding to $M_r \sim 66,000$ of the EPOR protein was present in all samples (Fig. 1A). Immunocytochemical labeling of both U87 and HT100 cells has also confirmed EPOR expression on the cell surface (Fig. 1B). Thus, our results are in agreement with recent studies showing that the EPOR is expressed in nonhematopoietic tissues (7–9).

One difficulty in studying the effects of exogenous rhEPO in cancer cells is inconsistency of the results. Whereas we could clearly show the existence of EPOR in the cancer cell lines studied in the current work, we could not consistently show an effect on the corresponding cellular response to rhEPO. The effect of exogenous rhEPO was observed to vary from one batch of FBS to another and that the level of erythropoietin as measured with the anti-erythropoietin antibody indicated the level of erythropoietin in FBS is low (100 milliunits/mL). The discrepancy in

the finding indicates that although the bovine erythropoietin may be functionally active as the human erythropoietin with the cell line, it might not be immune-crossreact with the anti-erythropoietin antibody used in the erythropoietin detection method. A functional assay of the erythropoietin activity in the serum may be a more reliable measurement of the endogenous erythropoietin activity in the FBS. It has been previously shown that K562 cells cultured in growth medium supplemented with 10% FBS possess constitutively phosphorylated STAT5, whereas incubation of the cells in serum-free medium for 24 hours reduced the base level of STAT5 tyrosine phosphorylation (41–43). Moreover, treatment of serum-starved K562 cells with erythropoietin 30 units/mL of rhEPO lead to an increase in the tyrosine phosphorylation of STAT5, suggesting that presence of rhEPO in the cultured medium was required for STAT5 tyrosine phosphorylation. Although the STAT5 protein is a substrate for the JNK2, which becomes activated in response to erythropoietin binding to EPOR (44, 45), other extracellular signaling proteins were shown to be involved in JAK-STAT signaling (19). Nevertheless, because erythroid cell differentiation of K562 cells was shown to be erythropoietin dependent, it allowed us to utilize this cell line to determine the factor(s) behind the inconsistency of our early results. The effect of serum heat inactivation and addition of rhEPO to the growth medium on K562 cells erythroid differentiation were analyzed in different batches of the FBS. Without heat inactivation, the FBS is very capable of induce K562 cells erythroid differentiation, indicating of high level of erythropoietin activity in the serum sample. On the other hand, heat inactivation of the FBS leads to a lost of K562 cells erythroid differentiation ability (Fig. 2) and that the addition of rhEPO to heat-inactivated serum led to a significant increase in K562 differentiation in a dose-dependent manner. No effect of rhEPO addition was seen in the samples where nonheat-inactivated FBS was used to prepare growth medium, suggesting that the presence of endogenous erythropoietin in noninactivated serum masked the effect of rhEPO in cell proliferation studies (Fig. 2). The results of nonheat-inactivated and heat-inactivated fetal bovine serum also indicate that *in vitro* investigation of erythropoietin effect on nonhematopoietic cells should be proceed with conditions setup for cytokines

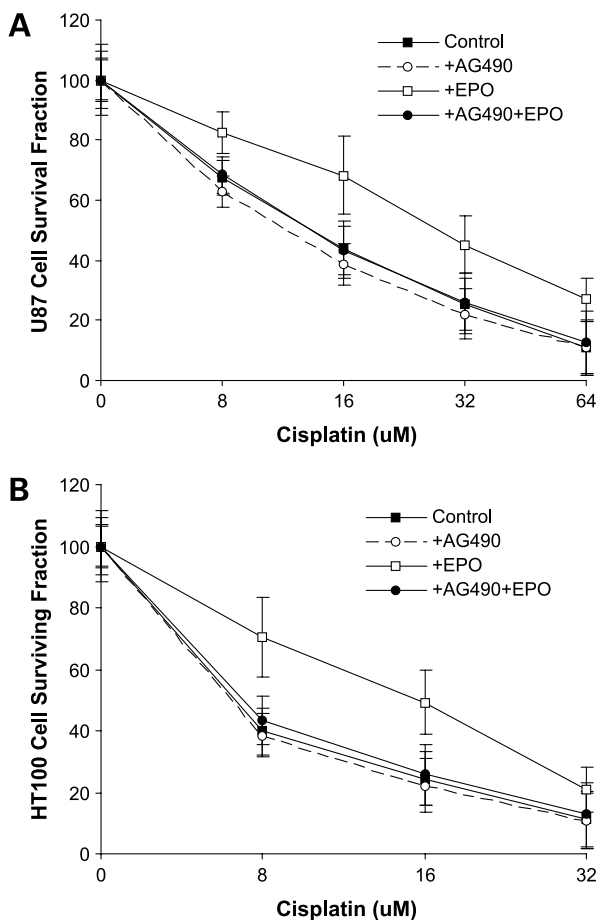


Figure 4. Survival of U87 and H100 cells as measured by the MTT assay after exposure to various doses of cisplatin. **(A)** Comparison of cisplatin sensitivity between untreated (Control), EPO-treated, tyrphostin-treated (AG490), and EPO + AG490-treated U87 cells. **(B)** Comparison of cisplatin sensitivity between untreated (Control), EPO-treated, tyrphostin-treated (AG490), and EPO + AG490-treated HT100 cells. Points, means from three independent experiments; bars, \pm SE.

such as IFN, where all assays are carried out using heat-inactivated FBS. It is important to reduce the level of endogenous erythropoietin in FBS before studying the effect of rhEPO in cell cultures.

We use two methods in measuring the cell survival, clonogenic assay for ionizing irradiation and MTT assay for cisplatin. The presence of rhEPO in the culture media increases the cell survival to cytotoxic agent cisplatin and to ionizing irradiation. As mentioned above, erythropoietin activates many signal transduction pathways. These include the JAK2/STAT5, the extracellular signal-regulated protein kinase pathway, and signal transduction molecules such as SHC/GRB-2, CRK-1, Vav, phosphatidylinositol 3-kinase, and the protein phosphatases SHP-1 and SHP-2 (23–27). Among the pathways, the JAK2 kinase has been shown to be an early effector of the EPOR signal transduction pathway (17) and seems to be the most specific for erythropoietin. Hence, we tested the effect of

the JAK2 kinase inhibitor, Tyrphostin (AG490), on the erythropoietin induced survival in U87 and HT100 cancer cells. Our experimental findings showed that the increased resistance of U87 and HT100 cells to ionizing radiation and to cisplatin after exposure to rhEPO can be abolished by the treatment with the specific JAK2 kinase inhibitor, tyrphostin (AG490; Figs. 3 and 4; Table 1). The effect of AG490 is specific to the erythropoietin-induced resistance, as the administration of AG490 alone did not affect U87 and HT100 cell sensitivity to ionizing radiation or to cisplatin, respectively. The result indicates that the signal transduction pathway is mediated by erythropoietin, although the signal transduction protein JAK2 in the human malignant glioma U87 and the primary cervical carcinoma cell lines HT100 is intact and functional in promoting cell growth and in inhibiting apoptosis induced by erythropoietin exposure. Recently, Liu et al. (46) tested the effect of both erythropoietin and granulocyte macrophage colony-stimulating factor in a series of cancer cells. They found that coculture with either the mitogen-activated protein kinase inhibitor U0126 or the granulocyte macrophage colony-stimulating factor antagonist E21R negated the induced resistance to cytotoxic chemotherapeutic agent cisplatin by granulocyte macrophage colony-stimulating factor. The result indicates that the mitogen-activated protein kinase signal transduction pathway mediated by the granulocyte macrophage colony-stimulating factor is intact in the tested cancer cells. It is not known, however, whether or not the JAK2 kinase is also involved, as this pathway was not tested. In the light of the result by Liu et al. (46), it is possible that the mitogen-activated protein kinase signal transduction pathway mediated by erythropoietin/EPOR might also be active in our cancer cell lines to provide resistance toward ionizing irradiation and to cisplatin. We are currently testing this possibility.

The presence of an intact erythropoietin pathway in cancer cells may help to explain the possible failure to show any benefit of disease control in the recent published clinical trials of erythropoietin in patients treated with radiation and/or chemotherapy (40, 47, 48). Our findings also indicate that the administration of rhEPO to cancer patients undergoing anticancer therapy should proceed with caution, especially when the cancer type has been shown to be positive for EPOR. Recently, Pinel et al. (49) showed that erythropoietin induces a reduction of hypoxia before and during fractionated irradiation. This resulted in an improvement of radioresponse in the human glioma xenografts (49). The status of the EPOR expression in the glioma cells used in the study, however, is unknown. The use of erythropoietin may be beneficial in improving the radiosensitivity and/or chemosensitivity in EPOR negative tumors. Perhaps erythropoietin should be considered to be a more general cytokine in its biological effect rather than a specific cytokine for RBC precursors. *In vivo* studies are planned to determine the effect of erythropoietin administration on the radiosensitivity and/or the chemosensitivity of EPOR positive and negative neoplastic cells in animal models.

Acknowledgments

We thank Sigat Choudhury, Paul Kauler, and Jean-Pierre Paiement for their helpful discussions.

References

- Kelleher DK, Matthiensen U, Thews O, Vaupel P. Tumor oxygenation in anemic rats: effects of erythropoietin treatment versus red blood cell transfusion. *Acta Oncol* 1995;34:379–4.
- Chapman JD, Engelhardt EL, Stobbe CC, Schneider RF, Hanks GE. Measuring hypoxia and predicting tumor radioresistance with nuclear medicine assays. *Radiother Oncol* 1998;46:229–37.
- Murray D, Mirzayans R, Scott AL, Allalunis-Turner M J. Influence of oxygen on the radiosensitivity of human glioma cell lines. *Am J Clin Oncol* 2003;26:E169–77.
- Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Res* 1996;56:4509–15.
- Graeber TG, Osmanian C, Jacks T, et al. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature* 1996;379:88–91.
- Westenfelder C, Baranowski RL. Erythropoietin stimulates proliferation of human renal carcinoma cells. *Kidney Int* 2000;58:647–57.
- Juul SE, Yachnis AT, Rojiani AM, Christensen RD. Immunohistochemical localization of erythropoietin and its receptor in the developing human brain. *Pediatr Dev Pathol* 1999;2:148–58.
- Juul SE, Zhao Y, Dame JB, Du Y, Hutson AD, Christensen RD. Origin and fate of erythropoietin in human milk. *Pediatr Res* 2000;48:660–7.
- Masuda S, Nagao M, Sasaki R. Erythropoietic, neurotrophic, and angiogenic functions of erythropoietin and regulation of erythropoietin production. *Int J Hematol* 1999;70:1–6.
- Acs G, Acs P, Beckwith SM, et al. Erythropoietin and erythropoietin receptor expression in human cancer. *Cancer Res* 2001;61:3561–5.
- Arcasoy MO, Jiang X, Haroon ZA. Expression of erythropoietin receptor splice variants in human cancer. *Biochem Biophys Res Commun* 2003;307:999–1007.
- Yasuda Y, Fujita Y, Masuda S, et al. Erythropoietin is involved in growth and angiogenesis in malignant tumours of female reproductive organs. *Carcinogenesis* 2002;23:1797–805.
- Acs G, Chen M, Xu X, Acs P, Verma A, Koch C. Autocrine erythropoietin signaling inhibits hypoxia-induced apoptosis in human breast carcinoma cells. *Cancer Lett* 2004;214(2):243–251. Also available from: <http://www.sciencedirect.com>.
- Spivak JL. Recombinant erythropoietin. *Annu Rev Med* 1993;44:243–53.
- Tilbrook PA, Klinken SP. The erythropoietin receptor. *Int J Biochem Cell Biol* 1999;31:1001–5.
- Watowich SS. Activation of erythropoietin signaling by receptor dimerization. *Int J Biochem Cell Biol* 1999;31:1075–88.
- Miura O, Nakamura N, Quelle FW, Witthuhn BA, Ihle JN, Aoki N. Erythropoietin induces association of the JAK2 protein tyrosine kinase with the erythropoietin receptor *in vivo*. *Blood* 1994;84:1501–7.
- Darnell JE Jr, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* 1994;264:1415–21.
- Liu KD, Gaffen SL, Goldsmith MA. JAK/STAT signaling by cytokine receptors. *Curr Opin Immunol* 1998;10:271–8.
- Witthuhn BA, Quelle FW, Silvennoinen O, et al. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. *Cell* 1993;74:227–36.
- Zhuang H, Patel SV, He TC, Niu Z, Wojchowski DM. Dominant negative effects of a carboxy-truncated Jak2 mutant on Epo-induced proliferation and Jak2 activation. *Biochem Biophys Res Commun* 1994;204:278–83.
- Chin H, Nakamura N, Kamiyama R, Miyasaka N, Ihle JN, Miura O. Physical and functional interactions between Stat5 and the tyrosine-phosphorylated receptors for erythropoietin and interleukin-3. *Blood* 1996;88:4415–25.
- Devemy E, Billat C, Haye B. Activation of Raf-1 and mitogen-activated protein kinases by erythropoietin and inositolphosphate-glycan in normal erythroid progenitor cells: involvement of protein kinase C. *Cell Signal* 1997;9:41–6.
- Damen JE, Liu L, Cutler RL, Krystal G. Erythropoietin stimulates the tyrosine phosphorylation of Shc and its association with Grb2 and a 145-Kd tyrosine phosphorylated protein. *Blood* 1993;82:2296–303.
- Miura O, Miura Y, Nakamura N, et al. Induction of tyrosine phosphorylation of Vav and expression of Pim-1 correlates with Jak2-mediated growth signaling from the erythropoietin receptor. *Blood* 1994;84:4135–41.
- Klingmuller U, Wu H, Hsiao JG, et al. Identification of a novel pathway important for proliferation and differentiation of primary erythroid progenitors. *Proc Natl Acad Sci U S A* 1997;94:3016–21.
- Tauchi T, Damen JE, Toyama K, Feng GS, Broxmeyer HE, Krystal G. Tyrosine 425 within the activated erythropoietin receptor binds Syp, reduces the erythropoietin required for Syp tyrosine phosphorylation, and promotes mitogenesis. *Blood* 1996;87:4495–501.
- Belenkov AI, Paiement JP, Panasci LC, Monia BP, Chow TY. An antisense oligonucleotide targeted to human Ku86 messenger RNA sensitizes M059K malignant glioma cells to ionizing radiation, bleomycin, and etoposide but not DNA cross-linking agents. *Cancer Res* 2002;62:5888–96.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–54.
- McGuckin CP, Forraz N, Liu WM. Diaminofluorene stain detects erythroid differentiation in immature haemopoietic cells treated with Epo, IL-3, SCF, TGFβ1, MIP-1α and IFNγ. *Eur J Haematol* 2003;70:106–14.
- Lappin TR, Elder GE, Taylor T, McMullin MF, Bridges JM. Comparison of the mouse spleen cell assay and a radioimmunoassay for the measurement of serum erythropoietin. *Br J Haematol* 1988;70:117–20.
- Hoffman R, Murnane MJ, Benz EJ Jr, et al. Induction of erythropoietic colonies in a human chronic myelogenous leukemia cell line. *Blood* 1979;54:1182–7.
- Kitanaka A, Waki M, Kamano H, et al. Antisense src expression inhibits proliferation and erythropoietin-induced erythroid differentiation of K562 human leukemia cells. *Biochem Biophys Res Commun* 1994;201:1534–40.
- Neri LM, Bortol R, Tabellini G, et al. Erythropoietin-induced erythroid differentiation of K562 cells is accompanied by the nuclear translocation of phosphatidylinositol 3-kinase and intranuclear generation of phosphatidylinositol (3,4,5) trisphosphate. *Cell Signal* 2002;14:21–9.
- Spivak JL. Recombinant human erythropoietin and the anemia of cancer. *Blood* 1994;84:997–1004.
- Grogan M, Thomas GM, Melamed I, et al. The importance of hemoglobin levels during radiotherapy for carcinoma of the cervix. *Cancer* 1999;86:1528–36.
- Glaspy J. The impact of epoetin alfa on quality of life during cancer chemotherapy: a fresh look at an old problem. *Semin Hematol* 1997;34:20–6.
- Stuben G, Thews O, Pottgen C, Knühmann K, Vaupel P, Stuschke M. Recombinant human erythropoietin increases the radiosensitivity of xenografted human tumours in anaemic nude mice. *J Cancer Res Clin Oncol* 2001;127:346–50.
- Wun T, Law L, Harvey D, Sieracki B, Scudder SA, Ryu JK. Increased incidence of symptomatic venous thrombosis in patients with cervical carcinoma treated with concurrent chemotherapy, radiation, and erythropoietin. *Cancer* 2003;98:1514–20.
- Leyland-Jones B. Breast cancer trial with erythropoietin terminated unexpectedly. *Lancet Oncol* 2003;4:459–60.
- Carlesso N, Frank DA, Griffin JD. Tyrosyl phosphorylation and DNA binding activity of signal transducers and activators of transcription (STAT) proteins in hematopoietic cell lines transformed by Bcr/Abl. *J Exp Med* 1996;183:811–20.
- de Groot RP, Raaijmakers JA, Lammers JW, Jove R, Koenderman L. STAT5 activation by BCR-Abl contributes to transformation of K562 leukemia cells. *Blood* 1999;94:1108–12.
- Okutani Y, Kitanaka A, Tanaka T, et al. Src directly tyrosine-phosphorylates STAT5 on its activation site and is involved in erythropoietin-induced signaling pathway. *Oncogene* 2001;20:6643–50.

44. Penta K, Sawyer ST. Erythropoietin induces the tyrosine phosphorylation, nuclear translocation, and DNA binding of STAT1 and STAT5 in erythroid cells. *J Biol Chem* 1995;270:31282–7.
45. Sawyer ST, Penta K. Association of JAK2 and STAT5 with erythropoietin receptors. Role of receptor phosphorylation in erythropoietin signal transduction. *J Biol Chem* 1996;271:32430–7.
46. Liu WM, Powles T, Shamash J, Propper D, Oliver T, Joel S. Effect of haemopoietic growth factors on cancer cell lines and their role in chemosensitivity. *Oncogene* 2004;23:981–90.
47. Henke M, Laszig R, Rube C, et al. Erythropoietin to treat head and neck cancer patients with anaemia undergoing radiotherapy: randomised, double-blind, placebo-controlled trial. *Lancet* 2003;362:1255–60.
48. Lavey RS, Liu PY, Greer BE, et al. Recombinant human erythropoietin (EPO) as an adjunct to radiation therapy and cisplatin for stage IIB-IVA carcinoma of the cervix: A Southwest Oncology Group Study. *Int J Radiat Oncol* 2003;57:S190.
49. Pinel S, Barberi-Heyob M, Cohen-Jonathan E, et al. Erythropoietin-induced reduction of hypoxia before and during fractionated irradiation contributes to improvement of radioresponse in human glioma xenografts. *Int J Radiat Oncol Biol Phys* 2004;59:250–9.