Cancer Chemother Pharmacol (2008) 61:893–901
DOI 10.1007/s00280-007-0608-3

ORIGINAL ARTICLE

Recombinant erythropoietin differently affects proliferation of mesothelioma cells but not sensitivity to cisplatin and pemetrexed

Camilla Palumbo · Sonia Battisti · Daniela Carbone · Loredana Albonici · Maurizio Alimandi · Roberto Bei · Andrea Modesti

Received: 8 August 2007 / Accepted: 14 September 2007 / Published online: 6 October 2007

Abstract The combination of cisplatin and pemetrexed represents the newly established standard of care for patients with unresectable malignant mesothelioma (MM). However, this chemotherapy regimen appears to be associated with an increased prevalence of higher grade anemia as compared to treatment with cisplatin alone. Human recombinant erythropoietin (rHuEpo) is currently used for the treatment of anemia in cancer patients. Still, following the finding that the erythropoietin receptor (EpoR) is expressed by several tumor cells types and after the trials reporting that the recombinant cytokine can adversely affect tumor progression and patient survival, the clinical safety of rHuEpo administration to neoplastic patients has recently been questioned. The observation that the expression of EpoR, variably associated with the expression of the cognate ligand, is a common feature of MM cells prompted us to investigate whether treatment with rHuEpo could elicit proliferative and cytoprotective signals in EpoR-positive MM cell lines. Biochemical responsiveness of MM cells to rHuEpo was demonstrated by the time-course activation of both ERK1/2 and AKT following treatment with the recombinant cytokine. A moderately increased mitogenic activity was observed in two out of five MM cell lines treated with pharmacologically relevant concentrations of rHuEpo. On the other hand, the recombinant cytokine, administered either before or after cisplatin and pemetrexed, failed to interfere with the cytotoxic effects exerted by the chemotherapeutic drugs on the five MM cell lines. According to the presented findings, rHuEpo appears to have an overall limited impact on cell growth and no effect on MM sensitivity to chemotherapy.

Keywords Mesothelioma · Erythropoietin receptor · Epoetin beta · Cisplatin · Pemetrexed

C. Palumbo (✉) · S. Battisti · D. Carbone · L. Albonici · R. Bei · A. Modesti
Department of Experimental Medicine and Biochemical Sciences, Università degli Studi di Roma "Tor Vergata", Via Montpellier 1, 00133 Rome, Italy
e-mail: camilla.palumbo@uniroma2.it

M. Alimandi
Department of Experimental Medicine, Università "La Sapienza", Viale Regina Elena 324, 00161 Rome, Italy

Introduction

Malignant mesothelioma (MM) is a tumor originating from the mesothelial linings of the pleura and, less frequently, peritoneum and pericardium [1]. Traditionally considered a rare cancer, MM is showing an ever-increasing worldwide prevalence due to its strong association with asbestos exposure [2]. The aggressive and invasive properties of MM make it seldom amenable to surgical intervention, while its low radiosensitivity limits the efficacy of radiation therapy. In addition, until recently, MM response rates to conventional chemotherapeutic drugs or drug combinations were rarely reported to exceed 20%. In this regard, a systemic review of the literature indicated cisplatin as the most active single drug against MM [3]. Of late, a standard fist-line treatment for patients with unresectable MM has been established, based on the combination of cisplatin with the antifolate pemetrexed and yielding response rates of about 40% [2, 4, 5]. In spite of this significant achievement, MM remains a tumor with poor prognosis, and median survival from diagnosis is still less than 2 years.

Anemia is a well-known side effect of platinum-based chemotherapy, which can cause damage to both erythroid

progenitors and renal erythropoietin (Epo)-producing cells [6]. The combination of cisplatin and pemetrexed appears to be associated with an increased prevalence of higher grade anemia as compared to treatment with cisplatin alone [4, 5]. Human recombinant erythropoietin (rHuEpo) preparations are currently used for the treatment of anemia related to cancer therapies. However, the finding that several tumor cells types express the erythropoietin receptor (EpoR) has raised concern about the clinical safety of rHuEpo supplementation in neoplastic patients, on account of the growth-stimulatory and cytoprotective effects potentially exerted by the recombinant cytokine on the EpoR-bearing tumors [7–9]. This concern has been fostered by the results of two randomised, placebo-controlled trials, aimed at investigating the effects of rHuEpo in chemotherapy-treated patients with metastatic breast cancer [10] and in radiotherapy-treated patients with head and neck cancer [11], reporting decreased patient survival and increased tumor progression, respectively, in the rHuEpo-supplemented arms. Despite the methodological limitations found in these studies [12], it is remarkable that the reported negative impact of rHuEpo on head and neck cancer locoregional progression appeared restricted to patients with EpoR-expressing tumors [13]. However, the role played by the Epo/EpoR system in cancer growth, progression and response to therapy has yet to be clearly established and further reasearches are warranted in both preclinical and clinical settings [8, 9, 14].

In this report we provide evidence for the first time that expression of the EpoR is a common feature of pleural MM cells and show that it is associated with variable expression of the cognate ligand. Furthermore, we investigate whether the treatment with rHuEpo could elicit proliferative and cytoprotective signals in EpoR-positive MM cells and interfere with the cytotoxic effects of cisplatin and pemetrexed.

Materials and methods

Cell culture and reagents

Reagents for routine cell culture procedures were obtained from Euroclone (Milan, Italy). Previously characterized human pleural MM cell lines H-Meso-1 [15], MM-B1 and MM-F1 [16], Mero-25 and Mero-48a [17] were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin (complete medium). HepG2 and MCF-7 cells were maintained in RPMI 1640 supplemented as above. TF-1 cells were cultured in RPMI complete medium containing 10 ng/ml of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; R&D Systems, Minneapolis, MN, USA). The effects of exogenous Epo on MM cells were evaluated using the clinical grade rHuEpo epoetin beta (NeoRecormon; Roche, Mannheim, Germany). A research grade rHuEpo (R&D Systems) was also used in proliferation studies. Cisplatin (*cis*-platinum(II) diammine dichloride) from Sigma-Aldrich (St Louis, MO, USA) and pemetrexed (Alimta) from Eli Lilly and Company (Indianapolis, IN, USA) were used in MM survival studies.

Assessment of rHuEpo biological activity

During the course of the study, the biological activity of rHuEpo preparations was monitored using the growth factor-dependent TF-1 leukemic cell line [18]. TF-1 cells were seeded in 24-well plates in the absence of GM-CSF; after 24 h, fresh medium additioned or not of rHuEpo (1 IU/ml) or GM-CSF (10 ng/ml) was added to the wells and, at 48 and 96 h from treatments, cell proliferation was quantified by trypan blue staining and cell count.

Immunoblot analysis

Protein samples were resolved by SDS-PAGE, transferred to PVDF membranes (Hybond-P; Amersham Pharmacia Biotech, Little Chalfont, UK) and probed with the following primary antibodies: rabbit anti-EpoR (M-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-Epo (H-162; Santa Cruz), mouse anti-β-actin (clone AC-15; Sigma-Aldrich), rabbit anti-phospho-p44/42 MAP kinase and rabbit anti p44/42 MAP kinase (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-phospho-AKT (Ser473) and rabbit anti-AKT (Cell Signaling Technology). After incubation with the appropriate peroxidase-conjugated secondary antibodies, the immune complexes were visualized using an ECL detection system (SuperSignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL, USA). The specificity of EpoR labeling was verified by preadsorption of the M-20 antibody with a ten-fold excess of the corresponding immunizing peptide. Densitometric analysis of autoradiographic films was performed using the Image J software (National Institutes of Health, USA).

Evaluation of Epo synthesis and secretion

Near-confluent MM and HepG2 cultures were grown for 48 h in the presence of 10% FBS or under serum starvation. Conditioned media were then collected and clarified by centrifugation, while the cells were lysed. Culture replicates were trypsinized and used for cell counts. The cytokine released in the conditioned media was quantified using a commercially available ELISA kit (IBL, Hamburg, Germany) with a lower detection limit of 2.5 mIU Epo/ml.

Media from serum-starved cultures were further assayed after concentration to one-tenth of the original volume by Centricon filtration (10 kDa cut-off membrane; Millipore, Bedford, MA, USA). Parallel evaluation of Epo levels in cell lysates was carried out by western blotting; lyophilized human serum (IBL) was reconstituted and used as a positive control of immunostaining. To evaluate the effect of hypoxic stress conditions on Epo release, the cells were grown for 48 h in the presence or absence of the hypoxia-mimetic agent cobalt chloride (Sigma-Aldrich) at a 100 μM concentration. Conditioned media were then collected, clarified and subjected to ELISA.

Sulforhodamine B assay

MM cell proliferation and survival were quantified by the sulforhodamine B (SRB) assay, carried out as previously described [19]. The optical density (OD) of SRB-stained cultures was measured by an automated spectrophotometric plate reader at a wavelength of 490 nm. The assays were at first performed on samples containing twofold serial dilutions of cells, in order to determine the range of linearity between cell number and OD values.

MM cell proliferation studies

MM cells were seeded at low density in 96-well plates, allowed to adhere overnight, serum starved for 6–8 h and then switched to media additioned or not of rHuEpo (1–10 IU/ml) and FBS (2–10%). Media and treatments were renewed at 48 h and cell proliferation was measured after a total of 96 h. The experiments were performed in quadruplicate and repeated a minimum of three times.

Survival studies

The effects of epoetin beta (10 IU/ml) on survival of MM cells exposed to cisplatin (1–30 μM) or pemetrexed (10–1,000 nM) were investigated using two schedules of treatment differing in the sequence of administration of the recombinant cytokine versus the cytotoxic drugs. In both the schedules, MM cells were seeded in complete medium in 96-well plates at a density of about 7.5×10^3 cells/well for cisplatin/epoetin beta studies and at a density of about 3.0×10^3 cells/well for pemetrexed/epoetin beta studies. The first schedule of treatment was as follows: MM cells, allowed to adhere for 4–6 h, were treated with epoetin beta for 24 h and then switched to medium (200 μl) containing fresh cytokine and cisplatin or pemetrexed. The outline of the reverse schedule was: 24 h after cell seeding, medium (100 μl) containing cisplatin or pemetrexed was added to the individual wells followed, after additional 24 h, by medium (100 μl) containing epoetin beta. Control cultures were left untreated or treated with epoetin beta alone or either drug alone in the appropriate sequence. Cell survival was evaluated by SRB staining after a total of 72 h from cisplatin treatment and after a total of 96 h from pemetrexed treatment. The percentage survival of the cultures treated with cisplatin or pemetrexed was calculated by normalization of the OD values to those of the cultures receiving no cytotoxic drug. The percentage survival of the cultures treated with each drug plus epoetin beta was calculated by normalization of the OD values to those of the cultures treated with epoetin beta alone. The experiments were performed in triplicate and repeated three times.

Statistical analysis

Statistical significance of the observed differences was calculated by the Student's two-tailed *t* test.

Results

Expression of EpoR and Epo in MM cell lines

EpoR expression was investigated by western blot analysis in a panel of human pleural MM cell lines with epithelial (H-Meso-1, Mero-25), biphasic (MM-B1, Mero-48a) and sarcomatous (MM-F1) features, using the MCF-7 breast carcinoma cell line as positive control [7]. EpoR was expressed in all MM cell lines analyzed and its levels of expression were similar among MM cells and comparable to those MCF-7 cells (Fig. 1a, upper panel). The specificity of the immunostaining was verified by preincubation of the anti-EpoR antibody with a tenfold excess of the corresponding immunizing peptide (Fig. 1a, lower panel).

Next, Epo expression in MM cell lysates and its secretion into matched conditioned media were investigated by western blotting and ELISA, respectively, using the HepG2 hepatocellular carcinoma cell line as positive control. Indeed, HepG2 cells are known to synthesize and secrete large amounts of this cytokine [20]. As determined by western blot analysis, the cytokine was expressed at high levels, comparable to those of HepG2, in Mero-25 cells only, while much lower levels of expression were observed in the other MM cell lines (Fig. 1b). Serum starvation of MM and HepG2 cultures did not affect Epo expression in the corresponding cell lysates (data not shown). As determined by ELISA, HepG2 cells grown in the presence or absence of serum released 43 ± 3 and 24 ± 2 mIU of Epo per ml of medium, respectively. Conversely, in spite of its presence in the corresponding cell lysates, Epo was not detectable in the conditioned media of MM cells grown either in complete medium or serum-starved. Furthermore,

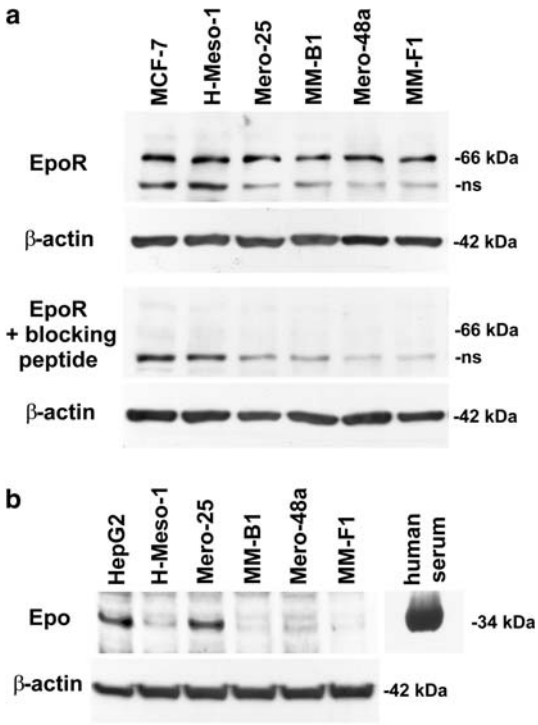

Fig. 1 Expression of EpoR and Epo in MM cell lines with epithelial (H-Meso-1, Mero-25), biphasic (MM-B1, Mero-48a) and sarcomatous (MM-F1) histotype. **a** *Upper panel* immunoblot analysis of EpoR expression in MCF-7 and MM cell lysates (40 μg/lane). *Lower panel* unlike non-specific band staining (*ns*), staining of the EpoR-specific band is inhibited by preadsorption of the primary antibody with a ten-fold excess of the corresponding immunizing peptide. After stripping, the membranes were reprobed with anti-*β*-actin to ensure equal loading and transfer of samples. **b** Immunoblot analysis of Epo in HepG2 and MM cell lysates (100 μg/lane) and, as additional control of the immunostaining, in a sample of human serum. This last is presented in a separate insert since the exposure times required to detect Epo in cell lysates resulted in overexposure of the corresponding Epo band

media from serum-starved MM cultures lacked Epo immunoreactivity even when concentrated to one-tenth of the original volume. Taking into account that Mero-25 cell lysates showed levels of Epo comparable to those of HepG2 and that similar numbers of cell equivalents were contained in the media of the two cell lines, the lack of Epo immunoreactivity in the media of Mero-25 cells is remarkable, and indicates that Epo secretion is at least poorly efficient in these cells. Finally, in order to determine whether the release of Epo by MM cells could be stimulated by hypoxic stress conditions, conditioned media of cultures grown for 48 h in the presence of the hypoxia-mimetic agent cobalt chloride were also tested by ELISA. Under cobalt chloride treatment, the Epo content of HepG2 conditioned media increased about 3.8-fold as compared to that measured in media from untreated control cultures, in agreement with previous reports [20], while the cytokine remained undetectable in MM cell media.

Assessment of biochemical responsiveness of MM cells to rHuEpo

According to the current literature, the treatment of EpoR-positive non-hematopoietic cancer cells with rHuEpo is not consistently effective in activating EpoR-linked signaling pathways [8, 21, 22]. Furthermore, it has been reported that a predominant cytosolic localization of the EpoR could account for the apparent lack of biochemical activity of rHuEpo on some cancer cell types [14, 23]. In order to define the biochemical responsiveness of EpoR-expressing MM cell lines to exogenous Epo, we investigated whether rHuEpo treatment of MM cultures could activate downstream effectors of the proliferative and cytoprotective signals elicited by the activated EpoR, namely the extracellular signal-regulated kinase 1 and 2 (ERK1/2 or p44/42 MAP kinases) and AKT [24, 25]. To this end, H-Meso-1, MM-B1 and MM-F1 cells, representative of the three main tumor histotypes, were serum-starved for 24 h, stimulated with rHuEpo (epoetin beta) at the pharmacologically relevant concentration of 10 IU/ml [22, 23] and lysed at different time points over a time course of 90 min from stimulation. MM cell lysates were then subjected to immunoblot analysis with antibodies that recognize phosphorylated epitopes of ERK1/2 and AKT. The results reported in Fig. 2a–c show that epoetin beta was effective in promoting the phosphorylation of ERK1/2 in MM cells. Kinetics and amplitude of ERK1/2 activation were similar in the three cell lines, with a peak activation occurring between 5 and 15 min from epoetin beta stimulation, followed by a decline toward basal levels. When investigating the effect of epoetin beta on AKT, basal levels of AKT phosphorylation were found in serum-starved H-Meso-1 and MM-B1, but not in MM-F1 cells (Fig. 2b), consistent with the marked differences in the basal as well as inducible activity of this kinase previously observed in distinct MM cell lines [26]. Upon epoetin beta treatment, the extent of AKT phosphorylation increased in H-Meso-1 and MM-B1, with slightly different kinetics but similar overall amplitudes (Fig. 2b–d). Conversely, the status of AKT activation was not apparently modified in MM-F1 cells (Fig. 2b). Altogether, these results indicate that rHuEpo treatment elicits signaling responses in EpoR-positive MM cells.

Effect of rHuEpo on MM cell proliferation

To investigate whether proliferation of EpoR-positive MM cell lines could be modulated by the administration of exogenous Epo, all five cell lines were grown under continuous exposure to 1–10 IU/ml of epoetin beta over a period of 96 h. Cell proliferation was then assessed by the SRB method. The assays were performed either in the absence of serum or, on account of a possible mitogenic synergism

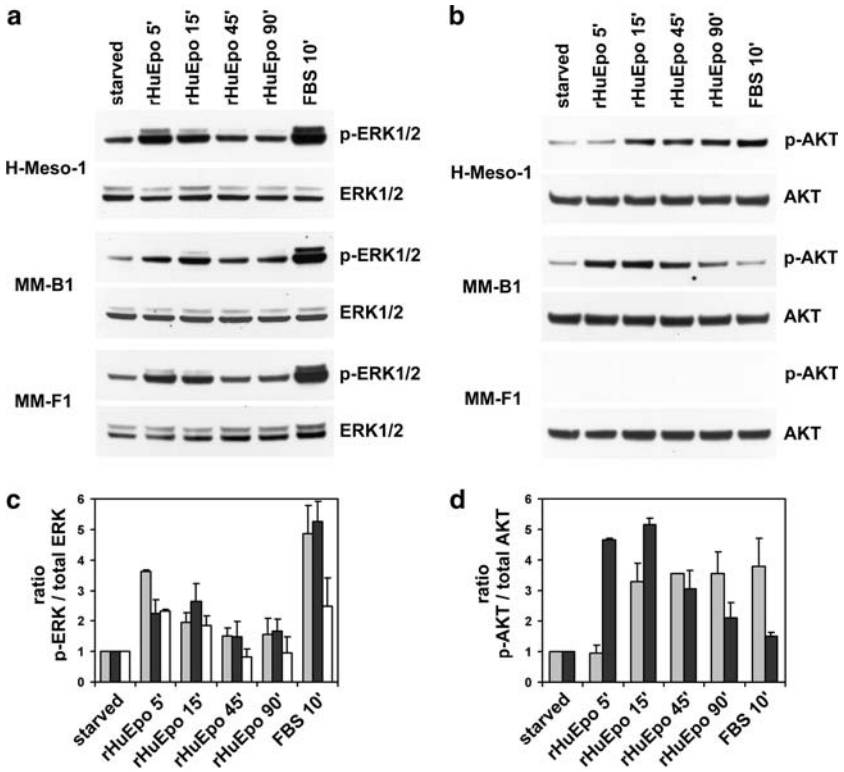

Fig. 2 Time courses of rHuEpo-induced ERK1/2 and AKT activation in epithelial (H-Meso-1), biphasic (MM-B1) and sarcomatous (MM-F1) cell lines. Serum-starved MM cells were treated with 10 IU/ml of rHuEpo (epoetin beta) for the indicated times and, for comparison, with 10% FBS for 10 min. **a–c** Representative western blots. The membranes were probed with anti-phospho-ERK1/2 or with anti-phospho-AKT and, after stripping, with anti-ERK1/2 or anti-AKT. **b-d** Ratios of p-ERK/total ERK and p-AKT/total AKT, as calculated after densitometric analysis of autoradiographic films. Shown are mean ± SE values from two to three different time course experiments performed with H-Meso-1 (*gray bars*), MM-B1 (*black bars*) and MM-F1 (*white bars*) cell lysates. The ratios are expressed in arbitrary units versus the values of serum-starved cultures

between rHuEpo and serum-contained growth factors or cytokines [27], in the presence of low (2%) and high (10%) serum concentrations. The treatment with epoetin beta exerted moderate but statistically significant mitogenic effects on H-Meso-1 and Mero-48a (Fig. 3a), whereas the growth of Mero-25, MM-B1 and MM-F1 was not affected by the recombinant cytokine under any of the tested conditions. The mitogenic activity of epoetin beta increased with decreasing serum concentration on both H-Meso-1 and Mero-48a cells. However, in the absence of serum epoetin beta exerted proliferative effects on H-Meso-1 but not on Mero-48a cells. In this regard, it is of note that Mero-48a cultures were the most sensible to serum depletion and, unlike the other MM cultures, displayed a high proportion of dying cells under prolonged serum starvation; nonetheless, this cell death was not rescued by epoetin beta supplementation. Proliferation of both the responsive cell lines was stimulated to a similar extent by 1 and 10 mIU/ml of epoetin beta, with an average gain versus the untreated cultures of about 20, 30 and 50% in H-Meso-1 cells grown in 10, 2 and 0% FBS, respectively, and of about 15 and 30% in Mero-48a cells cultured in the presence of 10 and 2% FBS.

Depending on their source, rHuEpo preparations can exhibit structural differences, mainly related to the presence and/or relative abundance of differentially glycosylated isoforms, which can impact on their biological properties and result in unequal activity on specific cell types [28–30]. Therefore, in order to rule out that the lack of mitogenic response observed in Mero-25, MM-B1 and MM-F1 cells could be related to the specific isoform composition of epoetin beta, proliferation assays on these cell lines were also performed using a different rHuEpo preparation (R&D Systems rHuEpo). The existence of structural differences between the two preparations of rHuEpo was verified by immunoblot analysis. As shown in Fig. 3b, the electrophoretic migration pattern of epoetin beta is characterized by a broader smear of different molecular weight products, consistent with the reported wide spectrum of isoforms contained in this preparation [29]. Nonetheless, as well as epoetin beta, the second rHuEpo preparation also failed to affect the proliferation of MM-B1, MM-F1 (Fig. 3c) and Mero-25 cells (data not shown). Finally, in order to exclude that the overall limited mitogenic effect exerted by HuEpo on MM cells could be due to a reduced biological activity

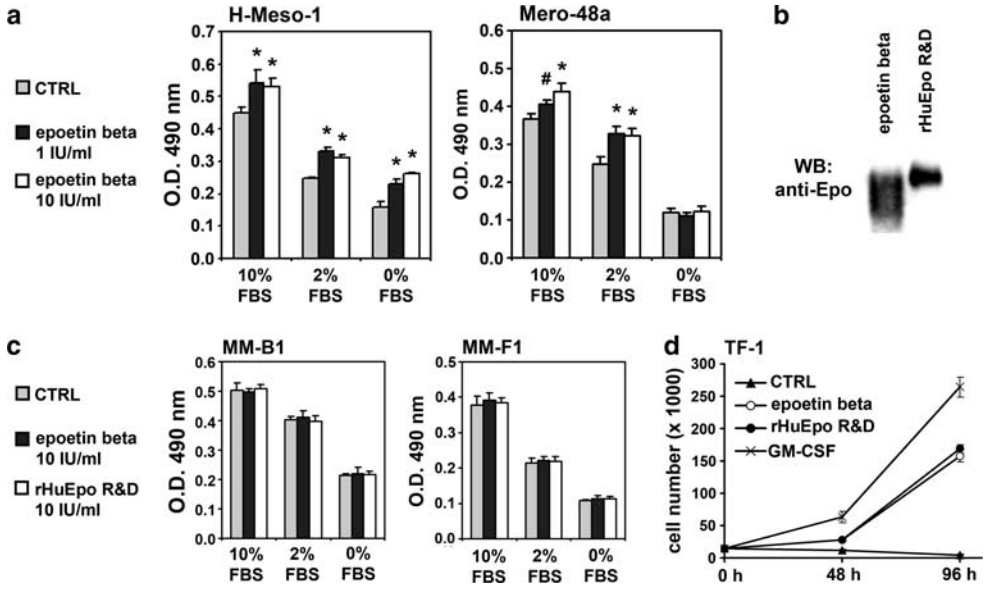

Fig. 3 Effect of rHuEpo on MM cell proliferation. MM cultures were subjected to SRB assay after 96 h of growth without (CTRL) or with rHuEpos, in the presence of 10, 2 and 0% FBS. **a** Treatment with epoetin beta (1–10 IU/ml) exerted a statistically significant effect on proliferation rates of H-Meso-1 and Mero-48a ($^{*}P < 0.05$ and $^{\#}P < 0.10$ vs. untreated controls, by Student's two-tailed *t* test). *Columns* mean from at least three different experiments, *bars* SE values. **b** Immunoblot analysis showing the electrophoretic migration patterns of epoetin beta and R&D Systems rHuEpo. **c** Treatment with either epoetin beta or R&D Systems rHuEpo (10 IU/ml) failed to modulate the growth rate of MM-B1 and MM-F1 cells. A similar lack of effect was observed on Mero-25 cells treated with 10 IU/ml of rHuEpos and on the three cell lines treated with 1 IU/ml of rHuEpos (not shown). *Columns* means from three different experiments, *bars* SE values. **d** Mitogenic activity of rHuEpo preparations on leukemic TF-1 cells. TF-1 cells were GM-CSF deprived for 24 h, treated with epoetin beta or R&D Systems rHuEpo (1 IU/ml) or, as control, with GM-CSF (10 ng/ml) and counted at the indicated time points

of the preparations here employed, the activity of both epoetin beta and R&D Systems rHuEpo was monitored by assessing their effect on the growth factor-dependent TF-1 leukemic cell line. Indeed, while long-term maintenance of TF-1 cells requires either GM-CSF or interleukin-3, their short-term growth and survival are sustained by Epo supplementation [18]. As shown in Fig. 3d, cell number of GM-CSF-deprived TF-1 cultures grown for 96 h in the presence of epoetin beta or R&D Systems rHuEpo was about 30-fold higher than that of GM-CSF-deprived control cultures. Thus, the strong activity of rHuEpos on TF-1 cells indicates that the overall limited impact exerted on MM cell growth is actually dependent on the specific responsiveness of MM cells.

Survival of MM cultures exposed to cisplatin or pemetrexed in the presence of rHuEpo

The potential modulatory effect of rHuEpo on MM cell sensitivity to chemotherapeutic drugs was investigated in survival assays performed on cells grown in the presence of epoetin beta (10 mIU/ml) and increasing concentrations of cisplatin (1–30 μM) or pemetrexed (10–1,000 nM). Furthermore, in order to unravel possible schedule-dependent cytoprotective effects of epoetin beta, the treatments were performed according to two schedules, differing in the sequence of administration of the recombinant cytokine versus the cytotoxic drugs, as detailed in "Materials and methods". The drug concentrations required to inhibit cell survival by 50% (IC_{50}) ranged from about 5 to 10 μM for cisplatin and from about 40 to 150 nm for pemetrexed, in agreement with previous reports [31–33]. The results obtained on all five cell lines indicate that epoetin beta does not affect MM chemosensitivity. Indeed, the recombinant cytokine failed to modify the survival of cisplatin- and pemetrexed-treated MM cells, either when administered before the cytotoxic drugs (Fig. 4) or when applied in the reverse sequence (not shown).

Discussion

In the present study, we provide evidence that expression of the EpoR is a common feature of MM cells cultured in vitro. Definite proof of EpoR expression by MM cells has not been previously reported to our knowledge, although the presence of binding sites for recombinant biotinylated Epo has been observed in tissue sections from one case of MM [34]. Several studies have described the expression and distribution of the EpoR in cancer tissues [7, 12, 13, 39, 47]. However, the suitability of commercial antibodies to detect the EpoR in vivo has recently been questioned due to

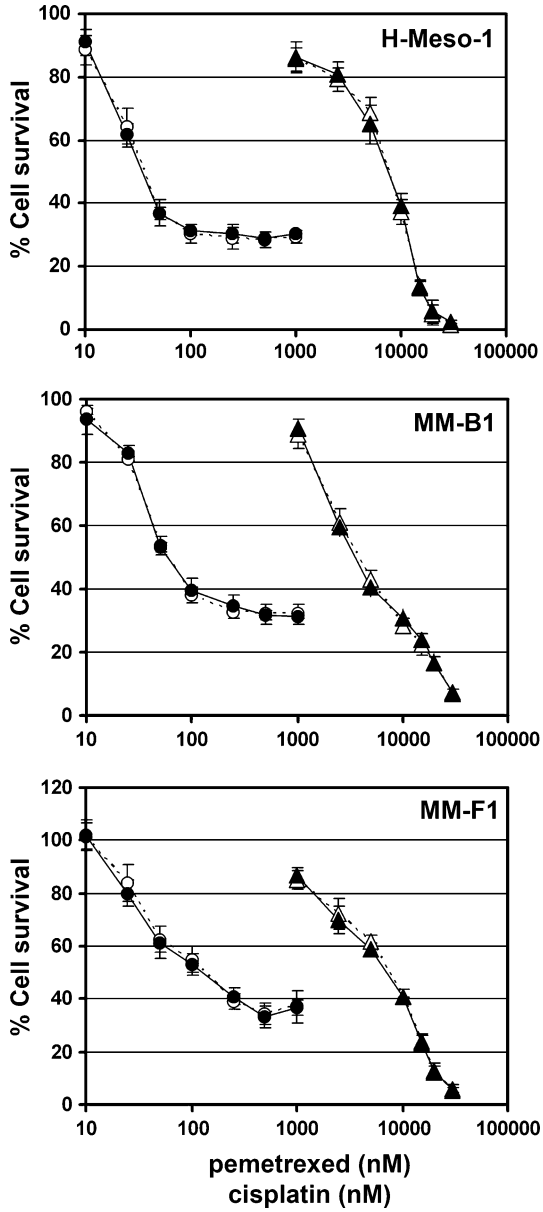

Fig. 4 Cell survival analysis. MM cultures were treated with epoetin beta (10 IU/ml) followed, after 24 h, by fresh cytokine and cisplatin or pemetrexed at the indicated concentrations. Control cultures were left untreated or treated with epoetin beta alone or either drug alone in the appropriate sequence. Survival of MM cultures treated with pemetrexed alone (*open circles/dashed line*) or pemetrexed and epoetin beta (*closed circles/solid line*) was evaluated after a total of 96 h from pemetrexed treatment; survival of MM cultures treated with cisplatin alone (*open triangles/dashed line*) or with cisplatin and epoetin beta (*closed triangles/solid line*) was evaluated after a total of 72 h from cisplatin treatment. Each data point represents the mean from three different experiments; *bars* SE values. Results analogous to those reported in the figure were obtained with Mero-48a and Mero-25 cells (not shown)

their poor specificity when used in immunohistochemical analysis [14, 35]. Therefore, more specific antibodies will be required to provide conclusive evidence and characterization of EpoR expression in MM tissues. Conversely, the expression of Epo by MM cells has been formerly demonstrated by an immunohistochemical study performed on tumor sections [36]. While our results on Epo expression by MM cells in vitro are corroborated by this study, we here report that albeit the cytokine is present at variable levels in MM cell lysates, it does not appear to be effectively released into MM culture media. Furthermore, lack of Epo immunoreactivity was observed in conditioned media of MM cultures grown under either standard conditions or hypoxic stress conditions generated by cobalt chloride treatment. The expression of Epo, as well as that of the cognate receptor, has been investigated in many tumor types both at the mRNA and protein level [12, 37], but the actual secretion of the cytokine by tumor cells has been demonstrated in a few studies only [20, 38, 39]. In this regard, our findings indicate that whether the Epo expressed by tumor cells could be effectively secreted and made available to activate autocrine or paracrine signaling pathways, is an issue which deserves further investigations, also on account of the reported angiogenic potential of tumor-derived Epo [28, 37, 40].

Similar to our finding of Epo intracellular retention in MM cells, a low ratio between cell surface and intracytoplasmic EpoR protein has been observed in different cell types [14]. Moreover, intracellular retention of the EpoR has been associated with the apparent lack of biochemical activity of rHuEpo on certain EpoR-positive cancer cells [23]. In fact, rHuEpo treatment of EpoR-positive non-hematopoietic cancer cells is not consistently effective in activating EpoR-linked signaling pathways [8, 21, 22]. Signal transduction by the EpoR, a receptor lacking intrinsic kinase activity, involves activation of the associated non-receptor tyrosine kinase JAK2 and of several downstream pathways [24, 25]. While the mitogenic action of Epo is thought to rely primarily on activation of the JAK2/STAT5 and RAS/ERK pathways, the PI3K/AKT pathway appears to have a main role in mediating Epo cytoprotective and antiapoptotic effects [9, 22, 37]. Our results showing the activation of both ERK1/2 and AKT upon epoetin beta stimulation demonstrate that rHuEpo is effective in eliciting signaling responses in MM cells. On the other hand, activation of these pathways upon rHuEpo treatment does not appear to be predictive of proliferative or survival responses by MM cells. Indeed, although ERK1/2 were activated to a similar extent and with similar kinetics in epoetin beta-treated H-Meso-1, MM-B1 and MM-F1 cells, proliferation was stimulated by the recombinant cytokine in H-Meso-1 cells only. Moreover, while epoetin beta was effective in activating AKT in both H-Meso-1 and MM-B1, it did not modify the cytotoxic effect exerted by cisplatin and pemetrexed on these cells. A similar lack of correlation between the activation of mitogenic and cytoprotective pathways and the effective induction of proliferative and

survival responses by rHuEpo has been previously observed in studies performed on different tumor cell types, and it is not clear whether it could be due to a limited amplitude and/or duration of the elicited signals or the parallel activation of counteracting molecular pathways [22, 41, 42]. Altogether, these previous studies and the results presented here highlight that the molecular determinants of tumor cell responsiveness to the mitogenic and cytoprotective actions of rHuEpo have yet to be clearly defined.

The treatment of EpoR-bearing tumors with rHuEpo has been reported to be ineffective in modulating cell proliferation or, conversely, to induce mitogenic activity [12, 14, 37]. These conflicting results could be related to differences intrinsic to the specific tumor types investigated. However, the validity of some of these studies is limited due to the use of suprapharmacological doses of rHuEpo [12, 14]. In the present study we used pharmacologically relevant concentrations of rHuEpo [14, 22, 23] and observed that cell proliferation was moderately but significantly increased in only two out of five EpoR-positive MM cell lines. In this respect our results indicate that the mitogenic activity of rHuEpo on EpoR-expressing tumor cells is cell-line specific rather than tumor type-specific. It is of note that we did not observe the previously reported mitogenic synergism between rHuEpo and serum [27] since, overall, the mitogenic activity of rHuEpo on both the responsive cell lines increased with decreasing serum concentrations. Remarkably, the proliferation of MM cells was stimulated to a similar extent by 1 and 10 mIU/ml of epoetin beta and the levels of growth stimulation, although much lower than those reported for erythroid cells [14, 18, 42], were comparable to those observed in other rHuEpo-treated cancer cell lines, including breast, renal, prostate and head and neck carcinomas [7, 27, 43, 44].

Contrasting results have also been reported with respect to the effects of rHuEpo on tumor cell sensitivity to chemotherapy, and in this regard rHuEpo has been found to have no effect as well as to enhance or reduce cell sensitivity to cytotoxic drugs [12, 14]. In the present study, we demonstrate that rHuEpo had no effect on MM cell sensitivity to cisplatin and pemetrexed, which are the drugs used as first-line standard regimen for patients with unresectable mesothelioma. Furthermore, our results indicate lack of schedule-dependent cytoprotective effects of rHuEpo, since the cytokine failed to modulate MM cell survival when administered either before or after the cytotoxic drugs.

While rHuEpo has been reported to interfere with the cytotoxic effect of cisplatin in cervical cancer, glioma and ovarian carcinoma cell lines and, conversely, have no effect on cisplatin activity in a panel of EpoR-positive malignant cell lines [45–48], to our knowledge this is the first study investigating the interaction between rHuEpo and pemetrexed. Remarkably, the lack of Epo immunoreactivity in MM cell media makes it unlikely that autocrine stimulation of the EpoR could have interfered with the activity of exogenously added Epo and lead to an underestimation of its mitogenic and cytoprotective potential on MM cells, and substantiates the results obtained in both cell proliferation and survival assays.

In conclusion, our data indicate that, although MM cells commonly express the EpoR and display biochemical responsiveness to rHuEpo, the administration of the recombinant cytokine appears to have an overall limited impact on cell growth and no effect on MM sensitivity to chemotherapy.

Acknowledgments We wish to thank Dr. Marjan Versnel, Department of Immunology, Erasmus Medical Center, Rotterdam, NL, for providing Mero-25 and Mero-48a cell lines. This work was supported by grants from the Italian Ministry of University and Research (MIUR-PRIN 2005) and the Italian Association for Cancer Research (AIRC REGIONALE 2005).

References

1. Robinson BWS, Lake RA (2005) Advances in malignant mesothelioma. N Engl J Med 353:1591–1603
2. Tsiouris A, Walesby RK (2007) Malignant pleural mesothelioma: current concepts in treatment. Nat Clin Pract Oncol 4:344–352
3. Berghmans T, Paesmans M, Lalami Y, Louviaux I, Luce S, Mascaux C, Meert AP, Sculier JP (2002) Activity of chemotherapy and immunotherapy on malignant mesothelioma: a systematic review of the literature with meta-analysis. Lung Cancer 38:111–121
4. Vogelzang NJ, Rusthoven JJ, Symanowski J, Denham C, Kaukel E, Von Pawel J, Gatzemeier U, Boyer M, Emri S, Manegold C, Niyikiza C, Paoletti P (2003) Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. J Clin Oncol 22:1589–1597
5. Hazarika M, White RM Jr, Booth BP, Wang YC, Ham DYL, Liang CY, Rahman A, Gobburu JVS, Li N, Sridhara R, Morse DE, Lostritto R, Garvey P, Johnson JR, Pazdur R (2005) Pemetrexed in malignant mesothelioma. Clin Cancer Res 11:982–992
6. Bohlius J, Weingart O, Trelle S, Engert A (2006) Cancer-related anemia and recombinant human erythropoietin—an updated overview. Nat Clin Pract Oncol 3:152–164
7. Acs G, Acs P, Beckwith SM, Pitts RL, Clements E, Wong K, Verma A (2001) Erythropoietin and erythropoietin receptor expression in human cancer. Cancer Res 61:3561–3565
8. Farrell F, Lee A (2004) The erythropoietin receptor and its expression in tumor cells and other tissues. Oncologist 9:18–30
9. Sytkowski AJ (2007) Does erythropoietin have a dark side? Epo signaling and cancer cells. Sci STKE 395:pe38
10. Leyland-Jones B, Semiglazov V, Pawlicki M, Pienkowski T, Tjulandin S, Manikhas G, Makhson A, Roth A, Dodwell D, Baselga J, Biakhov M, Valuckas K, Voznyi E, Liu X, Vervammen E (2005) Maintaining normal hemoglobin levels with epoetin alfa in mainly nonanemic patients with metastatic breast cancer receiving first-line chemotherapy: a survival study. J Clin Oncol 23:5960–5972
11. Henke M, Laszig R, Rube C, Schafer U, Haase KD, Schilcher B, Mose S, Beer KT, Burger U, Dougherty C, Frommhold H (2003) Erythropoietin to treat head and neck cancer patients with anaemia undergoing radiotherapy: randomised, double-blind, placebo-controlled trial. Lancet 362:1255–1260
12. Osterborg A, Aapro M, Cornes P, Haselbeck A, Hayward CRW, Jelkmann W (2007) Preclinical studies of erythropoietin receptor

expression in tumor cells: impact on clinical use of erythropoietic proteins to correct cancer-related anaemia. Eur J Cancer 43:510–519
13. Henke M, Mattern D, Pepe M, Bezay C, Weissenberger C, Werner M, Pajonk F (2006) Do erythropoietin receptors on cancer cells explain unexpected clinical findings? J Clin Oncol 24:4708–4713
14. Sinclair AM, Todd MD, Forsythe K, Knox SJ, Elliott S, Begley CG (2007) Expression and function of erythropoietin receptors in tumors. Cancer 110:477–488
15. Reale FR, Griffin TW, Compton JM, Graham S, Townes PL, Bogden A (1987) Characterization of a human malignant mesothelioma cell line (H-MESO-1): a biphasic solid and ascitic tumor model. Cancer Res 47:3199–3205
16. Catalano A, Romano M, Robuffo I, Strizzi L, Procopio A (2001) Methionine aminopeptidase-2 regulates human mesothelioma cell survival: role of Bcl-2 expression and telomerase activity. Am J Pathol 159:721–731
17. Versnel MA, Bouts MJ, Hoogsteden HC, van der Kwast TH, Delahaye M, Hagemeijer A (1989) Establishment of human malignant mesothelioma cell lines. Int J Cancer 44:256–260
18. Kitamura T, Tojo A, Kuwaki T, Chiba S, Miyazono K, Urabe A, Takaku F (1989) Identification and analysis of human erythropoietin receptors on a factor-dependent cell line, TF-1. Blood 73:375–380
19. Keepers YP, Pizao PE, Peters GJ, van Ark-Otte J, Winograd B, Pinedo HM (1991) Comparison of the sulforhodamine B protein and tetrazolium (MTT) assays for in vitro chemosensitivity testing. Eur J Cancer 27:897–900
20. Goldberg MA, Glass GA, Cunningham JM, Bunn HF (1987) The regulated expression of erythropoietin by two human hepatoma cell lines. Proc Natl Acad Sci USA 84:7972–7976
21. Westphal G, Niederberger E, Blum C, Wollman Y, Knoch TA, Rebel W, Debus J, Friedrich E (2002) Erythropoietin and G-CSF receptors in human tumor cells: expression and aspects regarding functionality. Tumori 88:150–159
22. Gewirtz DA, Di X, Walker TD, Sawyer ST (2006) Erythropoietin fails to interfere with the antiproliferative and cytotoxic effect of antitumor drugs. Clin Cancer Res 12:2232–2238
23. LaMontagne KR, Butler J, Marshall DJ, Tullai J, Gechtman Z, Hall C, Meshaw A, Farrell FX (2006) Recombinant epoteins do not stimulate tumor growth in erythropoietin receptor-positive breast carcinoma models. Mol Cancer Ther 5:347–355
24. Li F, Chong ZZ, Maiese K (2004) Erythropoietin on a tightrope: balancing neuronal and vascular protection between intrinsic and extrinsic pathways. Neurosignals 13:265–289
25. Richmond TD, Chohan M, Barber DL (2005) Turning cells red: signal transduction mediated by erythropoietin. Trends Cell Biol 15:146–155
26. Ramos-Nino ME, Vianale G, Sabo-Attwood T, Mutti L, Porta C, Heintz N, Mossmann BT (2005) Human mesothelioma cells exhibit tumor cell-specific differences in phosphatidylinositol 3-kinase/AKT activity that predict the efficacy of Onconase. Mol Cancer Ther 4:835–842
27. Westenfelder C, Baranowski RL (2000) Erythropoietin stimulates proliferation of human renal carcinoma cells. Kidey Int 58:647–657
28. Anagnostou A, Lee ES, Kessimian N, Levinson R, Steiner M (1990) Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells. Proc Natl Acad Sci USA 87:5987–5982
29. Storring PL, Tiplady RJ, Gaines Das RE, Stenning BE, Lamikanra A, Rafferty B, Lee J (1998) Epoetin alfa and beta differ in their erythropoietin isoform composition and biological properties. Br J Haematol 100:78–89
30. Yuen CT, Storring PL, Tiplady RJ, Izquierdo M, Wait R, Gee CK, Gerson P, Lloyd P, Cremata JA (2003) Relationship between the *N*-glycan structures and biological activities of recombinant human erythropoietins produced using different culture conditions and purification procedures. Br J Haematol 121:511–526
31. Kim JH, Lee KW, Jung Y, Kim TY, Ham HS, Jong HS, Jung KH, Im SA, Kim TY, Kim NK, Bang YJ (2005) Cytotoxic effects of pemetrexed in gastric cancer cells. Cancer Sci 96:365–371
32. Gordon GJ, Mani Madhubalan, Maulik G, Mukhopadhyay L, Yeap BY, Kindler HL, Salgia R, Sugarbaker DJ, Bueno R (2007) Preclinical studies of the proteasome inhibitor bortezomib in malignant pleural mesothelioma. Cancer Chemother Pharmacol, Epub ahead of print
33. Min SH, Goldman ID, Zhao R (2007) Caffeine makedly sensitized human mesothelioma cell lines to pemetrexed. Cancer Chemother Pharmacol, Epub ahead of print
34. Kayser K, Gabius HJ (1992) Analysis of expression of erythropoietin-binding sites in human lung carcinoma by the biotinylated ligand. Zentralbl Pathol 138:266–270
35. Elliott S, Busse L, Bass MB, Lu H, Sarosi I, Sinclair AM, Spahr C, Um M, Van G, Begley CG (2006) Anti-Epo receptor antibodies do not predict Epo receptor expression. Blood 107:1892–1895
36. Butnor KJ, Nicholson AG, Allred DC, Zander DS, Henderson DW, Barrios R, Haque AK, Allen TC, Killen DE, Cagle PT (2006) Expression of renal cell carcinoma-associated markers erythropietin, CD10, and renal cell carcinoma marker in diffuse malignant mesothelioma and metastatic renal cell carcinoma. Arch Pathol Lab Med 130:823–827
37. Hardee ME, Arcasoy MO, Blackwell KL, Kirkpatrick JP, Dewhirst MW (2006) Erythropoietin biology in cancer. Clin Cancer Res 12:332–339
38. Stolze I, berchner-Pfannschmidt U, Freitag P, Wotzlaw C, Rossler J, Frede S, Acker H, Fandrey J (2002) Hypoxia-inducible erythropoietin gene expression in human neuroblastoma cells. Blood 100:2623–2628
39. Kumar SK, Acs G, Fang D, Herlyn M, Elder DE, Xu X (2005) Functional erythropoietin autocrine loop in melanoma. Am J Pathol 166:823–830
40. Hardee ME, Cao Y, Fu P, Jiang X, Zhao Y, Rabbani ZN, Vujaskovic Z, Dewhirst MW, Arcasoy MO (2007) Erythropoietin blockade inhibits the induction of tumor angiogenesis and progression. PloS ONE 2:e549
41. Dunlop EA, Percy MJ, Boland MP, Maxwell AP, Lappin TR (2006) Induction of signalling in non-eyrthroid cells by pharmacological levels of erythropoietin. Neurodegener Dis 3:94–100
42. Erickson-Miller CL, Pelus LM, Lord KA (2000) Signaling induced by erythropoietin and stem cell factor in UT-7/Epo cells: transient versus sustained proliferation. Stem Cells 18:366–373
43. Feldman L, Wang Y, Rhim JS, Bhattacharya N, Loda M, Sytkowski AJ (2006) Erythropoietin stimulates growth and STAT5 phosphorylation in human prostate epithelial and prostate cancer cells. Prostate 66:135–145
44. Lai SY, Childs EE, Xi S, Coppelli FM, Gooding WE, Wells A, Ferris RL, Grandis J (2005) Erythropoietin-mediated activation of JAK-STAT signaling contributes to cellular invasion in head and neck squamous cell carcinoma. Oncogene 24:4442–4449
45. Acs G, Zhang PJ, McGrath CM, Acs P, McBroom J, Mohyeldin A, Liu S, Lu H, Verma A (2003) Hypoxia-inducible erythropoietin signaling in squamous dysplasia and squamous cell carcinoma of the uterine cervix and its potential role in cervical carcinogenesis and tumor progression. Am J Pathol 162:1789–1806
46. Belenkov AI, Shenouda G, Rizhevskaya E, Cournoyer D, Belzile JP, Souhami L, Devic S, Chow YK (2004) Erythropoietin induces cancer cell resistance to ionizing radiation and to cisplatin. Mol Cancer Ther 3:1525–1532
47. McBroom JW, Acs G, Rose GS, Krivak TC, Mohyeldin A, Verma A (2005) Erythropoietin receptor function and expression in epithelial ovarian carcinoma. Gynecol Oncol 99:571–577
48. Liu WM, Powles T, Shamash J, Propper D, Oliver T, Joel S (2004) Effect of haemopoietic growth factors on cancer cell lines and their role in chemosensitivity. Oncogene 23:981–990