Review article

Erythropoietin and the erythropoietin receptor, and drug resistance and progression of the neoplastic disease

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ABSTRACT

Preclinical studies conducted over the past 10 years have shown that EPO is not only a hormone that regulates erythropoiesis, a major growth factor, but also a cytoplasm with pleiotropic activity that also affects cancer cells. The expression of EPO and its receptor (EPOR) occurs in many cancers of various origins. The EPO/EPOR system is active in many cancer cells and is involved in the modification of molecular signaling pathways and the stimulation of growth, survival, motility and the ability to create metastases. EPO can also increase the resistance of cancer cells in vitro and in vivo to chemotherapy and radiotherapy.

Key words: erythropoietin, erythropoietin receptor, factors stimulating erythropoiesis, tumors, resistance to treatment, metastases

INTRODUCTION

Anaemia is a term derived from the Greek word *anaimia* (lack of blood), and consists in a reduced oxygen-carrying capacity of blood. As it often coexists with neoplastic diseases, it frequently compromises the course of therapy, negatively affecting the patient's quality of life. It occurs in ca. 40% of patients at the time of diagnosing a malignant neoplasm, and in over a half of patients undergoing anti-cancer treatment [1]. In cancer patients, anaemia compromises their quality of life, hinders and/or delays chemotherapy or radiotherapy, causes radioresistance of cells in radiotherapy, and has a negative prognostic value (in particular in patients suffering from lymphomas, head and neck tumours, lung cancer, cervical cancer and prostate cancer).

When treating cancer patients, substitution of deficiencies (iron, vitamin B_{12}, folic acid) is offered as well as packed red blood cell transfusions and erythropoiesis-stimulating agents (ESA). ESA drugs act by stimulating erythropoiesis receptors. The use of ESAs reduces the number of required transfusions, thus decreasing the risk of adverse transfusion reactions, and improving the quality of life (Cochrane Tonia 2012 metaanalysis) in patients with chemotherapy-induced anaemia.

Preclinical studies performed over the past 10 years demonstrated that erythropoietin (Epo) is not just an erythropoiesis-regulating hormone, and main growth factor, but also a cytokine with pleiotropic effects, also affecting cancer cells. Expression of Epo and its receptor (EpoR) has been found in neoplasms of different origins. The Epo/EpoR system is active in many neoplastic cells, and is involved in the modification of molecular signalling pathways as well as in the stimulation of growth, survival and metastatic potential [2, 3]. During more than 10 years of studies on the impact of Epo on the growth, invasiveness and treatment resistance of cancer cells, a number of phenomena have been discovered, whose mechanisms may not be explained solely with the help of the classical pattern of action of the receptor and its ligand, limited to a single cell type only. Presumably, important findings will be gathered based on the clinical implications of the following phenomena: EpoR constitutive activity, EpoR level of expression and functionality in the cells of specific tumour types, cross-interactions between the Epo/EpoR signalling pathway and tamoxifen, trastuzumab and platinum derivatives, and the indirect impact of Epo on the tumour in question, via its effect on the tumour microenvironment cells.

ANAEMIA AS A POOR PROGNOSTIC FACTOR IN ONCOLOGY, AND THE USE OF RECOMBINANT EPO

Anaemia is a poor prognostic factor in cancer, as it causes hypoxia (reduced tumour oxygenation is also present at the level of haemoglobin < 11 g/dl), which leads to chemo- and radioresistance, genetic instability, selection of clones resistant to cytostatic drugs and ionizing radiation, apoptosis disturbances, and stimulation of angiogenesis. It all facilitates diseases progression and development of distant metastases, leading to poorer prognosis.

As ESAs increase the concentration of haemoglobin (Hb), it was decided that erythropoiesis would be stimulated [4] in order to improve tumour oxygenation, expecting an overall improvement of the treatment results. However, the studies referred to demonstrated that ESAs had paradoxically adversely affected progression-free survival (PFS), and had led to increased mortality levels in that group of patients. It should be emphasised here that such use of ESA is currently an off-label one (in some of the studies, patients on ESAs were not even diagnosed with anaemia, as their baseline Hb concentration was 12.5 g/dl, and the target one was over 14 g/dl).

Therefore, it turns out that there is no simple correlation between tumour oxygenation and Hb concentration. The correlation appears to exist up to a certain point (Hb level of ca. 14.5 g/dl) [5], but increasing the Hb concentration beyond that point worsens the rheological properties of blood, paradoxically driving tumour oxygenation down. Hence, ESAs are now administered in line with the current guidelines only in palliative patients with chemotherapy-induced anaemia, whose Hb levels are 9–11 g/dl. The level of 12 g/dl should not be exceeded, though. ESA use is aimed at preventing blood transfusion, and improving the patient's quality of life.

PLEIOTROPIC PROPERTIES OF ERYTHROPOIETIN, AND ERYTHROPOIETIN RECEPTOR

Another problem is the fact that erythropoietin has pleiotropic properties, and Epo receptor is not only found on haematopoietic cells, but may also be present on cancer cells (Henke et al. 2006). It has been demonstrated that patients who received ESAs, and whose EpoR expression was positive, had poorer treatment outcomes and shorter PFS than those without EpoR expression. Thus, a hypothesis has been offered that Epo may stimulate cancer cells by binding with their receptor. However, 3 years later, the very same authors, using the same clinical mate-

rial of head and neck tumours (Head and Neck Cancer, Enhance Trial), looked into the EpoR mRNA expression, and found no correlation of EpoR with cancer progression or reduced PFS. They found no significant differences in terms of overall survival (OS) either. The role of Epo in the progression of cancer thus remains unclear. Increased mortality was observed in renal and oncological studies, with target Hb levels exceeding 13 g/dl. It should be noted, though, that if the effect of Epo in cancer patients was limited only to thromboembolic complications, we would observe reduced OS, with no reduction in PFS.

The gene responsible for EpoR is not an oncogene. In a great majority of tumours, no amplification of the EpoR gene is observed (amplification has only been observed in two erythroleukemic cell lines). To date, no EpoR activating point mutations have been described in any neoplasm. EpoR mutations are associated with hypersensitivity to Epo, and lead to polycythaemia [6], but they do not cause increased cancer incidence. It has not yet been resolved, whether and why the use of ESA may have an adverse impact on the course of cancer disease and time of survival. Still, a meta-analysis of 53 studies, published in 2009, demonstrated a statistically significant increase in mortality and/or cancer progression in patients treated for breast cancer, non-small-cell lung cancer, head and neck cancer, cervical cancer or lymphoma, who received ESAs.

In the available literature, it has been demonstrated that lack of tissue expression of Epo in the tumour does have an impact on the survival parameters, with it being an independent prognostic factor with a negative impact on survival [7]. 176 renal tumours (ccRCC) were tested. In 47 (26.7%) of them no Epo expression was found. Immunohistochemical expression of Epo was found to have an impact on overall survival (OS) and disease-specific survival (DSS). DSS in patients whose tissue was positive and negative for Epo expression was 85.3% and 76.1% ($p = 0.044$), respectively. In a multifactorial analysis, lack of Epo expression turned out to be a negative prognostic factor, adversely impacting both overall survival ($p < 0.001$) and disease-specific survival ($p < 0.001$).

It is known that the impact of Epo on tumour cells depends on the type of tumour, EpoR level of expression and functionality in its cells, tumour microenvironment (hypoxia), and type of anti-cancer treatment involved (both synergistic and antagonistic effects). Therefore, the following question is raised: do (and in what way) ESAs impact the proliferation and survival of cancer cells, and is a functional EpoR at all present on neoplastic cells? The first possible direct mechanism of how recombinant Epo might stimulate cancer disease progression consists in Epo affecting tumour cells via EpoR, thus leading to cancer cell proliferation. Another mechanism might be the microenvironment stimulation, i.e. stimulation of the endothelial cells, macrophages and fibroblasts, creating conditions that are conducive to cancer cell survival. A yet another, third possible mechanism consists in an indirect effect stemming from the stimulation of haematopoiesis and the immune system, and increased risk of thromboembolic complications.

Epidemiological studies on the risk of thrombosis indicated that the use of Epo was associated with stroke in patients with myelodysplastic syndrome (MDS), and with increased risk of all cardiovascular events in patients with multiple myeloma [8]. Direct impact of recombinant erythropoietin (rhEpo) on breast cancer cells was demonstrated in 2013 by Todaro et al., who isolated five lines of breast cancer stem-like cells (BCSC) from tumours of female patients [9, 10]. In all five lines, in vitro expression and activity of EpoR was observed, as was increased resistance to paclitaxel, doxorubicin and 5-fluorouracil. The isolated BCSC cells had functional characteristics of neoplastic stem cells: in immune-compromised mice, they were capable of developing tumours, emulating the histological structure and marker expression pattern from patient tumours. In vivo, rhEpo increased the resistance of primary tumours to doxorubicin and 5-fluorouracil, and the resistance of metastatic tumours to paclitaxel. RhEpo, administered to mice alone or in combination with paclitaxel, led to an increased mass of metastatic tumours.

DIFFERENT FORMS OF EPO RECEPTOR

The functional form of EpoR is a dimer, whose stimulation activates the Janus protein tyrosine kinase 2 (Jak2 kinase), and then causes phosphorylation of transcription and transduction proteins, which in turns leads to the inhibition of apoptosis and enhanced cellular proliferation. There are also other forms of the receptor present on the cells: the soluble form, the truncated form, and a more complex form, which might have cytoprotective properties (some publications suggest that role with reference to, for instance, the cells of the central nervous system).

The current confusion, related to the search for answers to the question of whether a functional form of EpoR exists, results from methodological differences. Presently, we have three methods at our disposal, with which we may measure EpoR:

- Reverse Transcriptase-PCR (Rt-PCR), serving to measure the mRNA concentration of EpoR – we do not look into the functional EpoR protein in such cases, as it would require us to isolate tumour cells from stromal cells

- Immunohistochemical method (IHC), making it possible to measure the EpoR protein expression, both surface and intracellular
- Western Blot (WB), enabling the measurement of the EpoR protein in the protein extract from the examined cells (proteins separated on gel).

The problem boils down to the fact that until recently there have been no specific antibodies which would only be targeting EpoR [11, 12]. That was one of the reservations expressed in the paper involving EpoR measurements in patients suffering from head and neck tumours. Polyclonal anti-EpoR C20 antibodies reacted with proteins whose molecular weight was ca. 70 kDa, i.e. they would bind non-specifically with other proteins, and the m20 antibody would detect the alleged EpoR even in mice deprived of the EPOR gene. Hence, we still lack scientific evidence for the fact that EpoR mRNA amplification occurs in cancer cells [13].

The role of the erythropoietin-producing hepatocellular A2 receptor (EphA2) in the process of neoplasia has been demonstrated in the available literature. EphA2 belongs to the family of receptor tyrosine kinases, which interact with cell ligands known as ephrins. EphA2 occurs in many types of solid tumours, and is reportedly overexpressed and performs a key role in oncogenic signalling. It may also be a therapeutic target [14].

REPORTS ON EPO AS AN AGENT THAT STIMULATS RESISTANCE TO ANTI-CANCER TREATMENT

Recent years brought along well-documented experimental findings on the impact of Epo on the resistance of malignant tumours to treatment. Congenital or acquired resistance to drugs, which may concern all cytostatic therapies, is often believed to be the reason behind treatment failure. Already back in 2001, it was observed that in normal breast gland cells, Epo and EpoR expression is not detectable, but it is found, and its levels increase along with the growing degree of malignancy [15]. EpoR expression was also found to be negatively correlated with OS and recurrence-free survival (RFS).

In 2009, Larsson et al. performed examinations in 382 pre-menopausal women, whose breast tumours were different in terms of their ER, PR and EpoR expression. The patients received ESAs. It transpired that EpoR concentration was of both prognostic and predictive value in the patients. In ER+ and/or PR+ patients, who had not been treated with tamoxifen (ER inhibitor in breast cancer cells), high EpoR expression was positively correlated with RFS, whereas in the tamoxifen-treated patients, it turned out to be unfavourable [16]. In patients with undetected ER, the level of EpoR expression had no impact on RFS duration. Thus, it was demonstrated that EpoR activity may be independent of the administration of exogenous Epo, and in one way or the other involved in the response to tamoxifen treatment. Perhaps it results from molecular communication between EpoR and ER signalling pathways. Those concerns were addressed by other studies that looked into the cellular mechanisms. They confirmed a constitutive (Epo-independent) EpoR activity, and molecular cooperation of the ER-α i EpoR receptors in the transmission of proliferation signals in breast cancer cells [17, 18].

In 2010, Liang et al. published their results on the antagonistic effects of EpoR and HER2 (human epidermal growth factor receptor) [19]. When testing the selected breast cancer cells, they also observed simultaneous expression of HER2 and EpoR. The use of trastuzumab (anti-HER2 antibody) inhibited HER2 in the activation of two of its molecular signalling pathways: PI3K (phosphatidylinositol 3-kinase)/Akt and MAPK (mitogen-activated protein kinases). When those cells were concurrently treated with trastuzumab and rhEpo, activity of the signalling pathways was restored. In vitro studies also demonstrated that rhEpo antagonizes trastuzumab-activated inhibition of cell growth, mobility and invasiveness. In vivo studies, on the other hand, indicated that breast tumour growth in mice was inhibited by trastuzumab, while rhEpo added to treatment to a large extent protected the tumours against the effects of trastuzumab. The observations were confirmed in a retrospective clinical study involving 111 patients with disseminated HER-2-positive breast cancer. The impact of rhEpo on the results of treatment with conventional chemotherapeutics was also looked into. A hypothesis was offered that perhaps rhEpo did not affect cancer cells themselves, but instead the microenvironment cells, thus creating conditions favourable for the survival of cancer cells. The hypothesis appears to be correct, as EpoR expression and activity was detected in epithelial cells.

What was very important was also the discovery of Epo's stimulating impact on the proliferation, invasiveness and drug-resistance of stem-like breast cancer cells [20]. Those cells are believed to be capable of self-renewal and differentiation, and to be responsible for disease recurrence following treatment. They are also inherently drug-resistant, and much more so than regular mature cancer cells.

In the available literature [21], it was found that in patients with decreased concentrations of Hb and normal creatinine levels, a progressive rise in Epo levels was also observed (only in pa-

tients undergoing platinum-based chemotherapy) since the first until the last chemotherapy cycle, and there were increased Epo levels following almost every platinum-based chemotherapy cycle (in particular in the midst of each cycle, which was probably caused by an increased sensitivity of the mechanism responsible for the release of Epo after the injection of a platinum agent, and then for its drop to the baseline level before the consecutive cycle). Renal efficiency was not correlated with Epo production. Several interpretations were proposed to explain the increase in Epo levels following cytostatic treatment:

- cytotoxic therapy that directly destroys Epo-producing cells in the kidneys simulates their hypoxia
- inhibition of bone marrow activity stimulates unknown factor to produce Epo
- reduction in erythrocyte precursors compromises the regular Epo degradation pathway.

A review of the available literature indicated that Epo was capable of inhibiting the cytotoxic effect of cisplatin in cervical cancer cells by activating anti-apoptotic responses regulated by the transcription factor signal transducer and activator of transcription 3 (STAT-3) [22]. The impact of Epo was assessed in three cervical cancer cell lines. It was observed that pre-incubation with Epo resulted in a significant reduction in cisplatin-induced apoptosis both in vitro and in vivo. Incubation with Epo induced expression and activation of STAT-3, which in turn stimulated expression and activation of the anti-apoptotic protein survivin. Cisplatin cytotoxicity was partially restored with the help of survivin – mY155. On the other hand, inhibition of STAT-3 activation with the use of sub-lethal doses of wP1066 did away with Epo's cytoprotective effect entirely. No statistically significant correlation was demonstrated between endogenous Epo levels and reaction to the treatment with recombinant human erythropoietin.

SUMMARY

For over 10 years of studies on the impact of Epo on the growth, invasiveness and treatment resistance of cancer cells, a number of phenomena have been revealed, whose mechanisms of action may not yet be thoroughly explained. Presumably, there may be significant clinical implications of the following factors: tumour type, treatment involved, form of EpoR receptor, constitutive activity of EpoR, synergism of EpoR and ER, and antagonism with HER-2, indirect impact of Epo on cancer cells via its effect on the tumour's microenvironment cells, and the impact of Epo on the patient's immune system and haematopoiesis.

It has not been proven so far that there is enhanced EpoR mRNA expression in cancer cells. The studies conducted to date have had some limitations due to the lack of a specific test, which seems no longer valid, as there are much more specific antibodies at the researchers' disposal at present. Further studies are on-going, aimed at clarifying the exact mechanism behind the increase in Epo blood levels following platinum-based chemotherapy.

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