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"Effects of Erythropoietin and Stem Cell Factor on normal and cancer cells: Implications for supportive therapy in oncological patients"

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Abstract and Introduction

Abstract

The ability of erythropoietin (Epo) to promote the production of red cells is currently exploited to treat chemotherapy-induced anemia. However, the expression of Epo receptor (EpoR) in a variety of cancer cells suggests that Epo-based supportive therapy can negatively affect the clinical outcome. In line with this hypothesis, some clinical trials have questioned the benefit of Epo administration in patients affected by different tumors, including breast cancer. In this study we directly determined the effect of Epo on cancer mammospheres, whose tumorigenic activity was validated through the establishment of xenografts in immunocompromized SCID mice.

Our preliminary data showed that EpoR was expressed in both, undifferentiated mammospheres and in differentiated primary breast cancer cells.

The presence of Epo increased the expansion and survival of tumor mammospheres and differentiated primary breast cancer cells. More importantly, Epo was able to considerably protect both, differentiated and undifferentiated breast cancer cells, from death induced by many anti-neoplastic drugs. Accordingly, we observed that Epo increased the expression of its receptor, induced activation of AKT/PKB and MAPKs and increased the expression of Bcl-xL in breast cancer cells. Thus, the use of Epo may promote the survival and growth of tumorigenic breast cancer cells by counteracting the cytotoxic effects of chemotherapy suggesting the need for alternative therapeutic options in cancer patients.

In a comprehensive investigation, 81/120 tumor types examined did not yield any sample positive for c-kit expression, suggesting that the use of Stem Cell Factor (SCF) should be safe in many of the most common malignancies. [1]

To determine the possible oncogenic effect of SCF, we compared the pro-tumor activity of Epo and SCF on breast cancer, the major cancer type in women. Among this, we tested the potential protective effects of SCF in preventing hematopoietic cell death during chemotherapy *in vivo.*

Our data are showing that Epo increased the expansion and survival of tumor mammospheres and differentiated primary breast cancer cells. More importantly, Epo was able to considerably protect both, differentiated and undifferentiated breast cancer cells, from death induced by many anti-neoplastic drugs possibly through increased expression of the anti-apoptotic protein Bcl-xL.

SCF, on the contrary, can not exert any pro-tumor activity, since the majority of cancer cells tested, particularly breast cancer, resulted negative for c-kit expression.

In *in vitro* experiments performed on primary human erythroid progenitors we found that SCF is able to prevent apoptosis of erythroid progenitors induced by promising new anticancer agents and *in vivo* SCF restores the density of bone marrow cells to the level of controls in mice treated with Cisplatin or 5-Fluorouracil (5-FU).

In peripheral blood analysis we observe an increase in the levels of all mature blood cells upon SCF administration. Therefore, taken together our experiments demonstrate that SCF protects the hematopoietic system from chemotherapy-induced damage *in vivo* and outline a protocol for a potential clinical application of SCF to prevent chemotherapy-induced cytotoxicity.

Introduction

Erytropoiesis is a dynamic and complex process that maintains the number of circulating erythrocytes within a relatively narrow range under changing physiologic conditions in normal individuals [2,3]. A reduction of this number it is known as anemia. In clinical oncology, anemia affects the majority of patients undergoing chemotherapy and it is related manly to inhibition of erythoid cell maturation in the bone marrow, tumor-associated bleeding, hemolysis, marrow damage from metastases or myelodysplasia and toxicities associated with chemotherapy and radiation therapy [4].In most cases, cancer-related anemia is thought also to be caused by a complex interaction between the tumor cell population and the immune system, which ultimately disrupts the normal erythropoiesis manly by reducing the production of Erythropoietin (Epo) from the kidney [2,3,5,6].Epo is the primary regulator of erythropoiesis, stimulating growth, preventing apoptosis and promoting differentiation of red blood cell progenitors by binding to its cognate cell surface receptor (EpoR). Therefore, in therapy it is widely used for the prevention and treatment of chemotherapy-associated anemia. Epo significantly increases haemoglobin (Hb) levels, reduces transfusion requirements, and improves quality of life, particularly by relieving fatigue[4] .Recent studies however, have shown that Epo has biological functions aside from regulating erythropoiesis and that many solid tumors express EpoR, raising concerns about the fact that Epo could stimulate the growth of indolent tumors and interfere with the action of antitumor therapies, either by enhancing tumor proliferation rates or interfering with apoptotic cell death (Fig. 1). Epo responsiveness has also been identified in capillary endothelial cells, indicating that Epo may have a role in tumor angiogenesis [7-12] .This drawback has raised doubts on the opportunity to use erythropoietin in the supportive care of cancer patients [13, 14] suggesting the need for alternative therapeutic options.

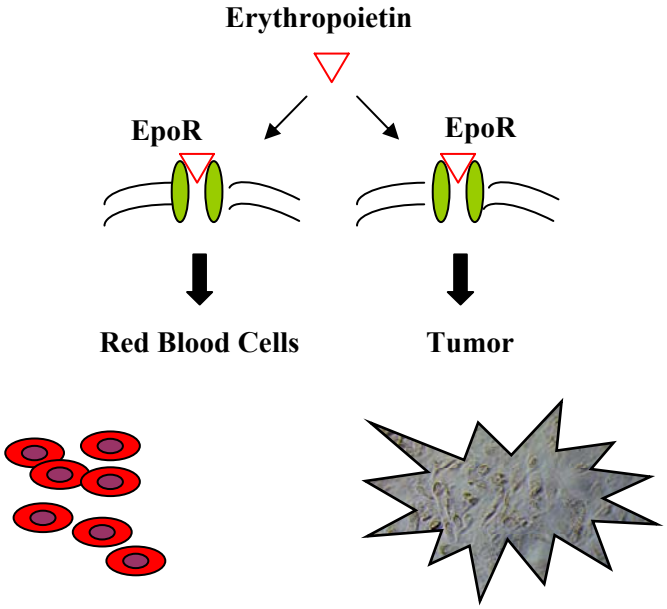

Figure 1.Possible mechanism of Erythropoietin. Epo stimulates red blood cell production by attaching to receptors on those cells. But there is mounting evidence that some nonhematopoietic cells also carry Epo receptors (EpoR). Susan Wolsborn.

Recently, the presence of c-kit, the receptor of an other essential factor for erythropoiesis, the Stem Cell Factor (SCF), has been extensively investigated in cancers of various origin. In a comprehensive investigation [1], 81/120 tumor types examined did not yield any sample positive for c-kit expression. C-kit negative tumors included both solid and hematologic tumors such as hormone-refractory prostate carcinoma, medulloblastoma, tubular breast carcinoma, Kaposi's sarcoma, non-Hodgkin's lymphomas and chronic myelogenous leukaemia.

Other investigators[15] reported a complete absence of c-kit expression in malignant brain tumors, breast and ovarian cancer, suggesting that the use of SCF should be safe in many of the most common malignancies.

Although SCF has not been used in clinic for the expansion of erythroid progenitors; studies have shown that SCF can cooperate with other cytokines such as G-CSF in long term cultures of human primitive hematopoitic cells as well as for ex vivo expansion of cord blood cells for transplantation[16] and in clinical trials in human with multiple myeloma, breast cancer and lymphoma SCF has been used successfully with G-CSF to mobilize stem and progenitors cells [17,18]. The c-kit receptor can be expressed by both normal and neoplastic tissues, where its stimulation by the natural ligand SCF can generate proliferative and survival stimuli. Therefore it is extremely important to avoid the use of SCF as a hemoprotective factor in oncologic patients expressing functional c-kit, as one could not rule out the possibility that it would enhance the growth and survival of the tumor itself. The risks related to the use of hematopoietic cytokines in the supportive care of cancer patients must also be carefully evaluated at the light of the recent discovery of cancer stem cells. The existence of a undifferentiated subpopulation of tumorigenic cells responsible for tumor maintenance, growth and spreading was known since several years in leukemias, but has been now demonstrated to occur also in solid tumors such as breast and brain cancers [19, 20]. The possibility that cancer stem cells may use hematopoietic growth factors to proliferate and resist to apoptotic stimuli poses an additional caveat for the administration of cytokines to cancer patients and requires a careful assessment of the presence of cytokine receptors on the surface of both stem and differentiated cancer cells. In our laboratory, we have obtained cancer stem cells from several solid tumors including breast, thyroid, colon, and lung carcinomas etc.

We analysed the expression of EpoR and c-kit in a variety of cancer types and we found that the large majority of stem and differentiated cancer cells tested resulted negative for c-kit expression and positive for EpoR expression. Above this, to determine the possible oncogenic effect of SCF, we compared the pro-tumor activity of Epo and SCF on breast cancer, the major cancer type in women.

Breast cancer cells, undifferentiated and differentiated, express EpoR, but do not express c-Kit, indicating that SCF can not have a pro-tumor effect on this type of cancer. In these cells SCF does not interfered with chemotherapy-induced toxicity. On the contrary the presence of Epo increased the expansion and survival of tumor mammospheres and differentiated primary breast cancer cells. More importantly, Epo was able to considerably protect both, differentiated and undifferentiated breast cancer cells, from death induced by many anti-neoplastic drugs possibly through increased expression of the anti-apoptotic protein Bcl-xL.

Thus, the use of Epo may promote the survival and growth of tumorigenic breast cancer cells while SCF should be safe in many of the most common malignancies and particularly in breast cancer.

Cancer related anemia occurs primarily due to progenitor cells loss.

In the normal hematopoietic system c-kit is expressed primarily by stem cells and progenitor cells and its expression decreases along with maturation in nearly all blood cells (with the exception of mast cells). Therefore, hematopoietic stem and progenitor cells represent the primary target of SCF, which acts by stimulating proliferation and inhibiting chemotherapy-induced cell death.

Therefore, we tested *in vitro* and *in vivo* the ability of SCF to act as an hemoprotective factor during chemotherapy treatment.

In *in vitro* experiments performed on primary human erythroid progenitors we found that SCF is able to prevent apoptosis of erythroid progenitors induced by CD95/Fas ligand and TRAIL, one of the most promising new anticancer agent, the death receptor ligand TRAIL (now entering phase II clinical trials). Moreover *in vivo*, by analysing bone marrow histological sections obtained from TRAIL-treated mice, we have found that TRAIL induces a moderate bone marrow toxicity (80% bone marrow cellularity of TRAIL-treated animals compared to 100% cellularity of control animals) and that simultaneous treatment with SCF restores the density of bone marrow cells to the level of controls. Thus, SCF may be useful to prevent blood cell depletion induced by TRAIL and possibly by other novel apoptosis-based anticancer agents.

In a different set of experiments, histological sections from mice treated with Cisplatin or 5-Fluorouracil (5-FU) with or without SCF show that mice treated with cisplatin or 5-FU display strong marrow hypoplasia with myelofibrosis and clusters of megakaryocytes. Treatment of mice with SCF resulted in both cases in a strong myeloprotection, as shown by high bone marrow cellularity and the almost complete disappearance of dysplastic cells.

Moreover, as a consequence of apoptosis inhibition of stem and progenitor cells exposed to chemotherapy, we observe an increase in the levels of all mature blood cells upon SCF administration.

Therefore, our experiments demonstrate that SCF protects the hematopoietic system from chemotherapy-induced damage *in vivo* and outline a protocol for a potential clinical application of SCF to prevent chemotherapy-induced cytotoxicity.

Erythropoietin and Stem Cell Factor

Role of Erythropoietin and Stem Cell Factor:

Erytropoiesis is a dynamic and complex process and its appropriate regulation is essential for embryonic development, adult red cell production and suppression of carcinogenesis.

Survival and apoptosis of hematopoietic stem/progenitor cells are crucial in maintaining the homeostasis of blood cell production and hematopoietic growth factors are crucial for controlling the balance between survival and apoptosis. It is well know that , among a number of growth factor, Stem Cell Factor (SCF) and Erythropoietin (Epo) are the two essential factor for Erythropoiesis [21-25]

Erythropoietin.

Erythropoietin is a glycoprotein hormone that serves as the primary regulator of erythropoiesis by stimulating growth, preventing apoptosis and inducing differentiation of red blood cell precursor [26].

Clinically, these actions translate into increased levels of haemoglobin, which has lead to the widespread use of recombinant human Epo (rHuEpo) in the treatment of patients with anemia due to renal failure , cancer or cancer therapy.

In humans, Epo mRNA encodes a protein 193 amino acids (aa) and loss of the C-terminal arginine during post-translation modification result in a 165-aa structure that comprises the mature protein (Fig.1).

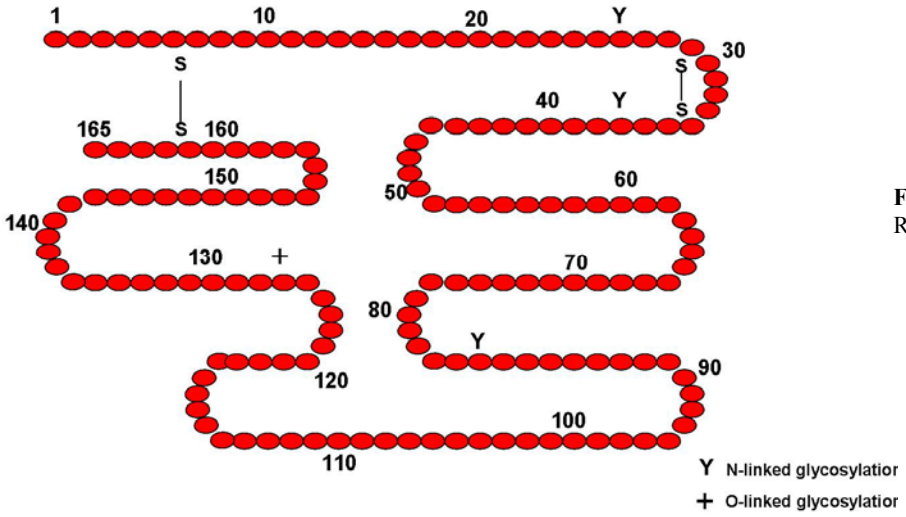

Figure 1. Structure of erythropoietin. Reprinted from Mulcahy.

The Epo molecules contains two structure-stabilizing disulfide bounds between aa 7 and 161 and 29-33, the reduction of which results in loss of bioactivity.

Additionally, the Epo molecules possesses three N-linked sugars, at position 24, 38 and 83, and one O-linked sugar at position 126. The O-linked sugar has no important function, but the N-linked sugars are necessary for stability of the Epo molecule in the circulation [27,28].

The Erythropoietin Receptor (EpoR).

The Epo receptor belongs to the cytokine receptor superfamily [29]. Included in this family are receptors for other hematopoietic growth factors, including growth hormone, prolactin, G-CSF, GM-CSF, thrombopoietin, oncostatin M, and several interleukins. Receptors in this family share several distinct features, including an extracellular ligand-binding domain with two pairs of conserved cysteine residues and a conserved motif, WSXWS, located close to the transmembrane domain; a single transmembrane domain; and an intracellular domain lacking catalytic activity (Fig. 2).

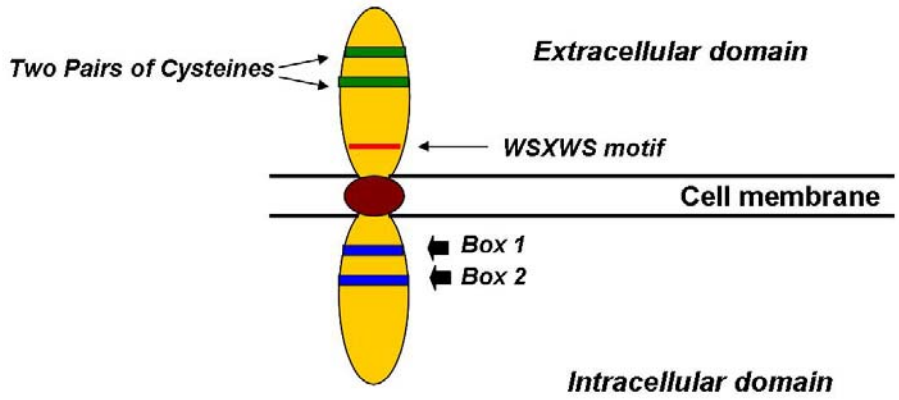

Figure 2. Schematic representation of The Epo receptor. The extracellular domains are anchored in the cell membrane. Mulcahy 2001.

EPO exerts its effects by inducing homodimerization of two molecules of the Epo receptors on the cell surface, which initiates the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signal transduction cascade that regulates cell proliferation and differentiation .

Unlike many other receptors, the EPO receptor has no intrinsic tyrosine kinase activity to activate receptor signaling. Rather, signaling appears to be mediated by JAK2, a cytoplasmic tyrosine kinase constitutively associated with the intracellular domain of the EPO receptor. JAK2 molecules associated with each of the individual EPO receptors are brought into close proximity, inducing their transphosphorylation and subsequent activation (Fig. 3). Activated JAK2 then phosphorylates several intracellular proteins, including the EPO receptor itself. The phosphorylated tyrosines act as docking sites for various intracellular proteins containing Src (tyrosine kinase) homology 2 domains, for example, one pathway activated is the JAK2/STAT5 pathway. Although the precise role of STAT5 in erythroid differentiation is not yet fully understood, JAK2-mediated STAT phosphorylation results in the formation of stable STAT dimers, which in turn translocate to the

nucleus where they bind to specific regulatory sequences and activate the transcription of target genes resulting in erythroid differentiation [28, 30, 31], in particular, STAT5 appears to mediate the induction of Bcl-xL by Epo. In addition to the STAT5 pathway, other signaling pathways, including RAS and PI3K, can be activated by EPO. PI3K signaling is believed to result in the activation of AKT and $p70^{s6K}$, which play a key role in transcription and cell-cycle progression. This PI3K–Akt pathway also leads to upregulation of Bcl-xL and inhibition of apoptosis. A further mechanism could be represented by NF-kB that is also a target of the PI3K-Akt pathway and mediates antiapoptotic signaling by platelet-derived growth factor EPO signaling may also activate many nonreceptor tyrosine kinases (e.g., c-fos/fes, $p72^{syk}$, and hematopoietic progenitor kinase-1), as well as proliferation-stimulating tyrosine phosphatases SHP2, SH2 inositol 5'-phosphatase, and other signal(Fig.3).

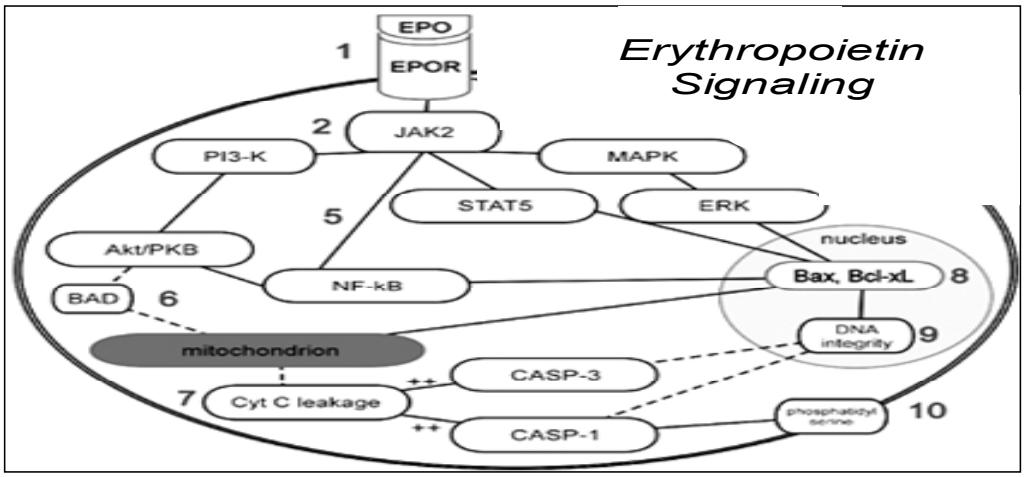

Figure 3.Summary of demonstrated EPO-signaling, Binding of EPO to its receptor leads to phosphorylation of janus kinase . This subsequently activates multiple cascades recruiting PI3-K, Stat5 and MAPKinase. Further, NF-kB is reported to be dually activated by JAK-2 and by Akt. The net effect is a reduction in the proapoptotic protein BAD and probability of mitochondrial leakage of cytochrome *C* ,an increased production of antiapoptotic proteins of the Bcl-x family and ultimate preservation of the DNA integrity. To the extent cytochrome *C* leakage is not prevented, caspase activation also occurs, inducing DNA degradation and the externalization of phosphatidyl serine on the cell membrane promoting the activation of the inflammatory cells. Solid lines indicate activation; dashed indicate inhibition

Stem Cell Factor

Stem cell factor is widely expressed during embryogenesis and can be detected in brain, endothelium, gametes, heart, kidney, lung, melanocytes, skin, and the stromal cells of the bone marrow, liver, and thymus [32]. SCF exists as membrane-anchored and soluble isoforms that arise from alternative RNA splicing and proteolytic processing; both isoforms contain initially an extracellular domain, a transmembrane segment, and an intracellular component [33].

The precursor for isoform 1, from which the soluble factor is derived, contains 273 amino acids. Residues 1–25 comprise the signal sequence, residues 26–214 make up the extracellular domain, residues 215–237 represent the transmembrane segment, and residues 238–273 constitute the intracellular component (the residue numbers in this article correspond to those of human proteins). Following the removal of the signal sequence, additional processing leads to the generation of the soluble form of SCF (residues 26–189). The enzyme that catalyzes the release of soluble SCF from isoform 1 is most likely matrix metalloprotease-9 [34]. Isoform 2 contains 28 fewer amino acids because exon 6 is omitted as a consequence of alternative splicing. In humans, isoform 2 lacks the metalloprotease-9 cleavage site and is chiefly membrane anchored.

Kit: The Stem Cell Factor Receptor.

Kit is a type III receptor protein-tyrosine kinase [35, 36] .The type III class also includes the platelet-derived growth factor (PDGF) receptor (α- and β-chains), the macrophage colony-stimulating-factor receptor (CSF-1), and the Fl cytokine receptor (Flt3). Receptor protein-tyrosine kinases all share the same topology: an extracellular ligand-binding domain, a single transmembrane segment, and a cytoplasmic kinase domain. The class III receptors are characterized by the presence of five immunoglobulin-like domains in their extracellular portion. Stem cell factor (SCF) binds to the second and third immunoglobulin domains while the fourth domain plays a role in receptor dimerization [33] . The structure of the class III receptors differs from that of other receptor tyrosyl kinases by the insertion of 70–100 amino acids near the middle of the kinase domain. In human Kit, the kinase insert is about 80 residues in length (Fig.4); this domain undergoes phosphorylation and serves as a docking site for a few pivotal signal transduction proteins.

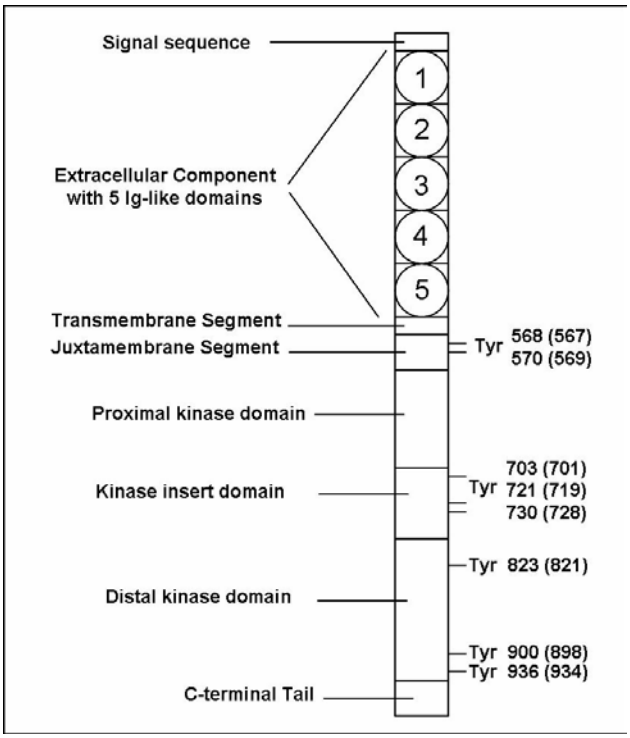

Figure 4. Organization of Kit. The relative length of the domains is to scale. The location of Kit gain-of-function mutations is indicated by the residue numbers on the right hand side of the figure. Ig, immunoglobulin; AL, activation loop.

Kit has the potential to participate in multiple signal transduction pathways as a result of interacting with several enzymes and adaptor proteins [37]. The adaptor protein APS, Src family kinases, and Shp2 tyrosyl phosphatase bind to phosphotyrosine 568. Shp1 tyrosyl phosphatase and the adaptor protein Shc bind to phosphotyrosine 570. C-terminal Src kinase homologous kinase (Chk) and the adaptor Shc bind to both phosphotyrosines 568 and 570. These residues occur in the juxtamembrane domain of Kit. Three residues in the kinase insert domain are phosphorylated and attract: (a) the adaptor protein Grb2 (Tyr703), (b) phosphatidylinositol 3-kinase (Tyr721), and (c) phospholipase Cγ (Tyr730). Phosphotyrosine 900 in the distal kinase domain binds phosphatidylinositol 3-kinase that in turn binds the adaptor protein Crk. Phosphotyrosine 936, also in the distal kinase domain, binds the adaptor proteins APS, Grb2, and Grb7 [37].The numerous Kit interactions cited above lead to activation of several signal transduction pathways (Fig.5) For example, phosphatidylinositol 3-kinase leads to the activation of Akt. Akt (protein kinase B), a protein-serine/threonine kinase, promotes cell survival [38].One substrate of Akt is Bad (Bcl2 antagonist of cell death), a pro-apoptotic protein that promotes cell death. Following phosphorylation, Bad no longer promotes apoptosis. Activation of the phosphatidylinositol 3-kinase/Akt pathway may explain in part how activating mutations of Kit participate in neoplastic transformation. Other downstream effectors of Kit include the Ras/mitogen-activated protein kinases and the Janus kinase/signal transducers and activators of transcription (Jak/STAT) pathways [39].

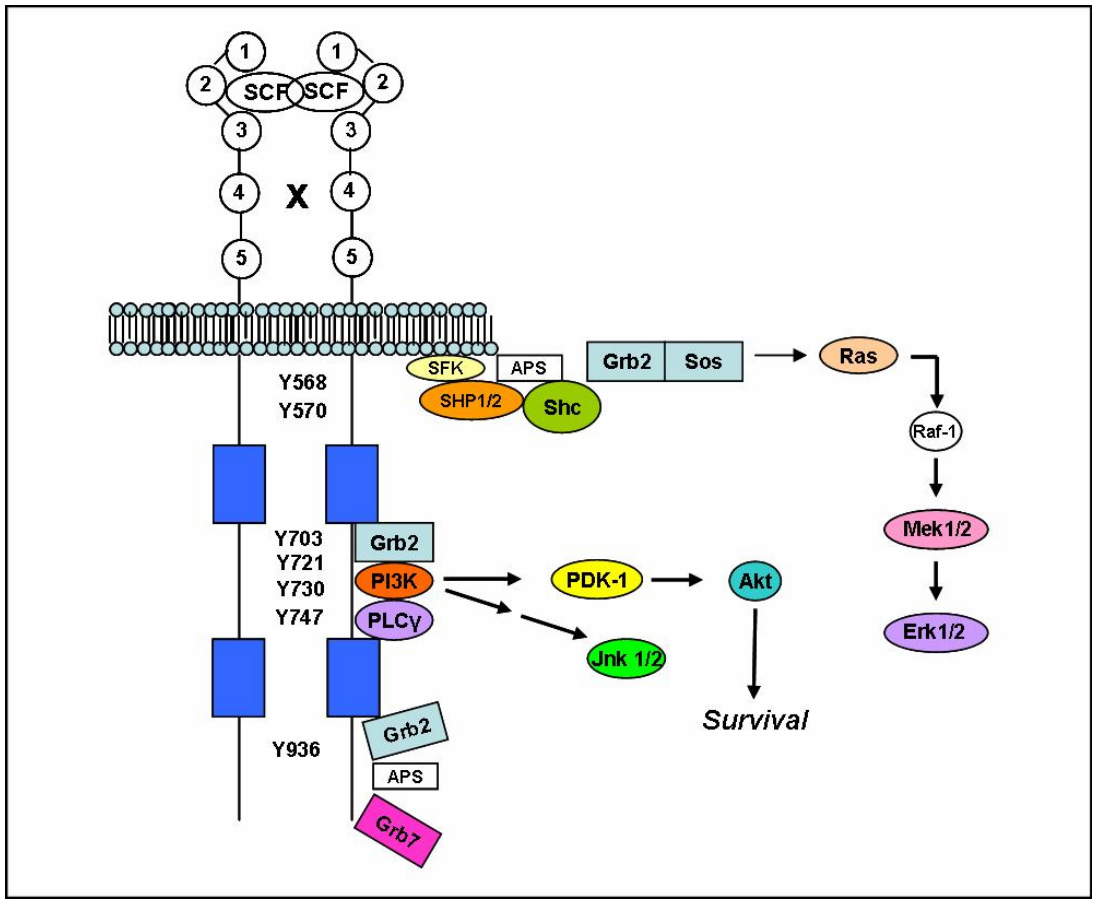

Figure 5. Schematic illustration of Kit interacting proteins. This figure summarizing signalling proteins activated by Kit and interaction sites on the receptor.

Mechanism of cooperation between c-Kit and erythropoietin receptor

Although SCF has been shown to induce some growth and survival of erythroid progenitors, its response is profoundly amplified in combination with Epo. Biochemical studies have provided evidence for physical association between c-Kit and Epo-R via the box 2 region cytoplasmic domain of the Epo-R [40]. Wu et al., utilizing various truncation mutants of Epo-R have demonstrated that c-Kit stimulation by SCF does not activate the Epo-R by inducing its dimerization, but by phosphorylating tyrosine residues in the cytoplasmic domain of the Epo-R [40]. Tan et al. have demonstrated that tyrosine residues 567 and 569 in the c-Kit receptor may play an essential role in regulating the phosphorylation of Epo-R as well as the synergy between c-Kit and Epo-R in erythroid cells [41].Specifically These authors demonstrated that SCF and Epo synergistically activate MAP kinase (Erk1/2), which correlates with cell growth and thus may be responsible for the synergistic effects observed in response to SCF and Epo co-stimulation in erythroid cells. Moreover, Kapur and Zhang utilizing an erythroid progenitor cell line G1E-ER2 cells demonstrated that c-Kit stimulation by SCF may play an essential role in the maintenance of Epo-R and Stat5 protein expression, which leads to increased expression of Bcl-xL and survival of erythroid progenitors in response to Epo stimulation [42]. In line with these observations, Sato et al. have shown an increase in the Epo-R mRNA in response to stimulation of HML/SE cells with SCF [43]. More recently, Boer et al. have shown that SCF can enhance Epo-mediated transactivation of Stat5 via the PKA/CREB pathway [44]. They showed that Epo induces transactivation of Stat5, which is enhanced by SCF treatment. SCF pre-treatment prior to Epo stimulation leads to a significant increase in Stat5 transactivation, however SCF stimulation alone did not affect Stat5 transactivation. The increase in Stat5 transactivation upon SCF pre-treatment was dependent on the PKA pathway. Since pre-treating the cells with the PKA inhibitors abrogated SCFs co-stimulatory effect. Biochemically, the downstream target of PKA, CREB showed increased activation after co-stimulation with SCF and Epo. Taken together, the results point to several distinct mechanisms of synergy between c-Kit and Epo-R in regulating normal erythroid cell development, with a major role for Stat5, Src family kinases and MAP kinases (Erk1/2). Whether all these mechanisms are operational in primary erythroid progenitors or whether the mechanisms are cell line specific remains to be determined.

Role of SCF and Epo in therapy

Anemia is a common symptom associated with most cancer patients, and appears in all patients with hematological malignancies [4].Cancer related anemia occurs primarily due to blood loss, bone marrow tumor infiltration, hemolysis, and folic acid deficiency [4].

In a recent European survey evaluating anaemia in over 13,000 patients with malignancies, anaemia was observed in 68% of patients at some time during the 6-month survey [45]. The frequency varied according to type of malignancy and treatment. Of patients with solid tumours receiving chemotherapy, the frequency of anaemia ranged from 62% in patients with gastrointestinal/colorectal tumours to 88% in patients with gynaecological tumours (Fig.6).

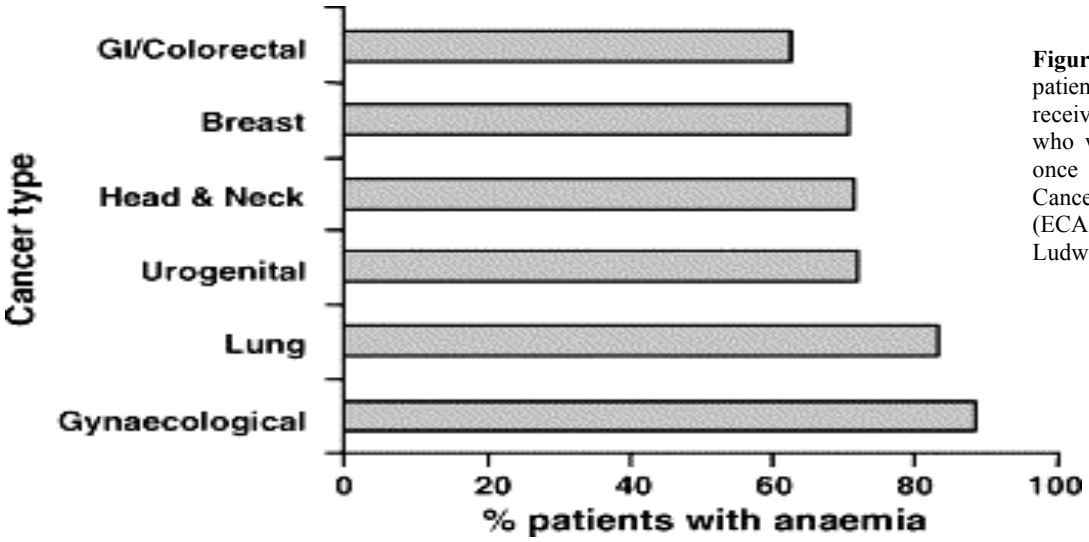

Figure 6. Percentage of patients with solid tumours receiving chemotherapy who were anaemic at least once during the European Cancer Anaemia Survey (ECAS) (adapted from Ludwig et al.

The manifestations of anaemia, including fatigue, dizziness, headache, shortness of breath, chest pain and depression, impact on the overall quality of life (QoL) of anaemic patients with cancer.

For more than a decade, recombinant human Epo(rhEpo) has been used as a therapeutic agent to treat anemia in adults with cancer. This type of treatment results in increased hemoglobin production, improves the quality of life and greatly reduces the need for blood transfusions [4, 46] .

In addition to its important role in the treatment of anemia associated with various diseases including cancer, human recombinant Epo has been shown to confer neutroprotective effects as well. In recent clinical studies, recombinant human Epo was shown to minimize tissue damage in patients with stroke.

The expression of Epo-R on cells of nervous system explains its role in neuroprotection functions [47, 48]. Recent studies suggest that a better understanding of the mechanism of action of Epo in the nervous system could allow the use of human recombinant Epo for treating patients with neurological disorders [49, 50].

Unlike Epo, SCF is not used in clinic for the expansion of erythroid progenitors although studies have shown that SCF can cooperate with other cytokines such as G-CSF in long-term cultures of human primitive hematopoietic cells as well as for ex vivo expansion of cord blood cells for transplantation [16].

In clinical trials in humans with multiple myeloma, breast cancer and lymphoma SCF has been used successfully with G-CSF to mobilize stem and progenitor cells [17, 18].

In some cases, co-administration of G-CSF and SCF compared to G-CSF alone in breast cancer patients has been shown to result in reduced level of tumor cell contamination and *in vitro* introduction of c-kit into a breast cancer cell line MCF-7 mediate inhibitory signals for the growth of breast cancer cells [51].

Mobilization of erythroid progenitors population is a major issue in cancer related anemia , in the normal hematopoietic system c-kit is expressed primarily by stem cells and progenitor cells and its expression decreases along with maturation in nearly all blood cells, with the exception of mast cells, Horsfall et al. [52] have demonstrated that the combination of G-CSF and SCF mobilizes the highest number of progenitor cells.

Recognition of SCF biological activity might therefore open new possibilities in the use of this factor in clinic.

Erythropoietin and Stem Cell Factor in cancer

Cytokines have assumed increasing importance in cancer biology with the demonstration that many can be produced by tumor cells and can influence the malignant process both positively and negatively. Cytokines may act on the cancer cells in an autocrine manner or on the supporting tissues, such as fibroblast and blood vessels, to produce an environment conducive to cancer growth [53]. More over, they can modulate several process involved in tumor progression and metastasis, for example, angiogenesis and the production of metalloproteinases.

The cellular receptor for SCF, c-kit, is detected in several normal and cancer tissues, but shows a restricted tissue distribution. In certain tumors, such as melanoma and breast cancer, the expression of c-kit diminishes as cell acquire a malignant phenotype [54], whereas small-cell lung cancer express c-kit rather frequently, often in combination with SCF [55].

A ligand-dependent activation of c-kit has been observed in gastrointestinal stromal tumors (GIST) as a consequence of mutations involving manly exon 11 [56-58] and, more rarely, exon 9 and 13 [58, 59].

The expression of c-kit in maintained only in some tumor histotypes, such as prostate cancer [60] ovarian and other gynaecological tumors [61-63] , gliomas [64, 65], neuroblastomas [66], small cell cancer and about half of non small cell lung cancers [67-70].

Conversely, its expression is frequently diminished or absent in cutaneous melanoma [71] and in breast [72-75] and thyroid cancers [76].

In particular, in breast tissue, some studies have shown that c-kit, widely expressed in normal epithelium, progressively decreases during malignant transformation and is present at low levels or

disappears in primary tumors and metastatic lesions [77]. Nishida et coll. showed that introduction of c-kit into a breast cancer cell line, MCF-7, mediate inhibitory signals for the growth of breast cancer cells [51]. c-kit then is expressed in a variety of normal cells and tissues, often in concomitance with its ligand, alternatively known as stem cell factor, mast cell growth factor, steel ligand, suggesting a role of these factors in the maintenance of a variety of fully differentiated tissues [78]. The interaction of c-kit with its ligand is essential in hematopoiesis, embryogenesis, proliferation and cellular migration, however since many studies have shown how c-kit expression progressively decreases during malignant transformation many authors are suggesting its role in maintaining normal growth rather then malignant transformation.

On the contrary, many studies have reported expression of EpoR in tumor cell lines as well as primary cancer suggesting that Epo might have pro-tumor effect by promoting growth and inhibiting apoptosis. Furthermore, the expression of EpoR in vascular endothelium in tumors has suggested potential effects of Epo on the tumor microenvironment, such as the stimulation of tumor angiogenesis. There is an accumulating body of experimental evidence for the presence of functional endogenus Epo-EpoR signalling in tumors from studies that used strategies to block Epo signalling pathways.

Yashuda et al. [79] reported that Epo signalling contributes to the growth and angiogenesis of female reproductive tract tumors. Blockade of Epo signalling with local soluble EpoR or anti-Epo antibody resulted in tumor cell destruction and reduction of vascularity in ovarian and uterine cancer xenografts, associated with an increased apoptotic death of both, tumor cells and vascular endothelial cells. It was also shown that injection of an EpoR antagonist blocked Stat5 phosphorylation and inhibited melanoma and stomach tumor cell survival and angiogenesis [80].

In another study, Arcasoy et al. [8] found that the administration of Epo-EpoR inhibitors in rat mammary adenocarcinoma tumors, resulted in significant tumors growth delay. These preclinical studies, taken together, suggest that the exploration of strategies to block Epo-EpoR function to target tumor growth and angiogenesis may be warranted.

Several studies reported Epo-modulation of tumor cell sensitivity to apoptosis induced by chemotherapy *in vitro* and *in vivo.* Batra et al. [11] showed increased expression of antiapoptotic genes (Bcl-XL, Bcl-2 and Mcl-1) as well as increase in Nuclear Factor-kB DNA binding activity in sarcoma and neuroblastoma cell line in response to rEpo. In human melanoma cells, incubation rEpo increased tumor cell resistance to hypoxia-induced cell death, moreover , rEpo increased cell viability during treatment with varying concentration of Cisplatin [81].

In august 2003 the Breast Cancer Erythropoietin Trial (BEST) was terminated early when researchers discovered a higher mortality rate in the Epo group than in those taking the placebo,

while Henke et al. demonstrated that in head and neck cancer Erythropoietin used to treat patients with anaemia was increasing tumor growth [82].

Taken together, these data suggest therefore that Epo may protect cancer cells against the effects of chemotherapy.

Material and Methods

Isolation, characterization and culture of primary and immortalized tumor cells. Primary culture of differentiated and undifferentiated cells from breast, ovary, lung, colon, kidney and thyroid carcinomas were established from specimens obtained from consenting patients undergoing surgery. Samples were obtained from Sant'Andrea Hospital (Rome) after approval by the local ethical committee. All the specimens were received within 2 hours from the surgery.

The tumors were cut up with scissors into small pieces and subsequently disrupted in the presence of Hanks' balanced salt solution (HBSS) and 150 mg/ml Collagenase I (Gibco) at 37° C for 1 -2 hours. Cell suspension was recovered, passed through 100 μm nylon cell strainer and subjected to Ficoll gradient centrifugation. Cells were washed twice with HBSS and finally cultured in DMEM medium supplemented with b-FGF (20 ng/ml), EGF (20 ng/ml) and Insulin (5 ng/ml).

For primary culture of differentiated cells, very low percentage (1%) of FBS was added to the medium.The immortalized cell lines MCF-7, SKOV, HT29 and H460 were cultivated in DMEM/F12 supplemented with 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin and 2 mmol/l L-glutamine and maintained in 5% CO_2 a 37° C.

TF-1 cell line was culture in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS.

HBSS, DMEM, DMEM/F12, FBS, penicillin, streptomycin and L-glutamine were all purchased from Life Technology Inc. (Grand Island, NY). IMDM was purchased from Euroclone (West York, UK). b-FGF, EGF and Insulin were purchased from Peprotech Inc. (Rocky Hill, NJ).

Flow cytometric analysis. To determine the level of expression of EpoR and c-kit one hundred thousand cells were used for flow cytometric analysis. Cells were washed with cold PBS and incubated with control or specific antibodies. Mouse anti-human EpoR antibody (R&D Systems, Minneapolis, MN), goat anti–human SCF Receptor/c-kit antibody (R&D Systems, Minneapolis, MN), phycoerythrin-conjugated anti-goat secondary antibodies (Chemicon, Temecula, CA), and FITC-conjugated anti-mouse antibodies (Molecular Probes, Eugene, OR) were used. Labeled cells were washed twice with PBS and fluorescence intensity was evaluated by FACScan (Becton Dickinson, San Jose, CA).

Confocal Microscopy. Primary differentiated and undifferentiated breast cancer cells, MCF-7 and TF-1 cell lines were fixed in 4% paraformaldehyde, permeabilized with PBS plus 0.1% of TRITON-X, washed 3 x with PBS and incubated O.N. at 4° C with primary antibodies anti-EpoR 0.2 μg/ml (BD Pharmingen), anti-c-kit 0.2 μg/ml (R&D Systems, Minneapolis, MN),and anti-NF.kB 0.2 μg/ml (Santa Cruz, CA), then washed with PBS 3 x, and incubated with secondary Abs. FITC-conjugated anti-mouse antibody (Molecular Probes, Eugene, OR) and Alexa Fluor® 488 goat

anti-rabbit IgG antibody (Molecular Probes, Leiden, Netherlands). were used. To detect Bcl-XL expression, undifferentiated breast cancer cells were expose to Epo 3U/ml for 24 h , fixed and permeabilized as described above and incubated with Anti-Bcl-X_L from Santa Cruz Biotechnology (Santa Cruz, CA) for 1 h RT. The cells were washed 3x and incubated with FITC-conjugated anti-mouse antibodies (Molecular Probes, Eugene, OR). Nuclei were stained in blue with Hoechst 33342® (Molecular Probes, Eugene, OR). Fluorescence images were taken by means of an Olympus FV-500 laser scanning confocal inverted microscope equipped with Argon ions, Green and Red Helium-Neon lasers and with PlanApo 40X dry, 60X and 100X oil Olympus objectives (numerical aperture 0.85, 1.4 and 1.35 respectively). Emission at different wavelengths was collected using the proper filters and overriding signal was subtracted. Digital zooming was applied where specified. Images were assembled with the Canvas 8 software (Deneba Systems Inc.).

Western blotting. For detection of EpoR (R&D Systems, Minneapolis, MN), phospho-ERKs (Cell Signaling Technology Inc., Beverly, MA), phospho-AKT (Cell Signaling Technology Inc., Beverly, MA), and Bcl-X_L (Santa Cruz, CA), MCF-7 cells were synchronized in PRF-SFM for 24h and subsequently exposed to Epo 3U/ml for other 24h. Protein extracts were prepared by resuspending cell pellets in 1% NP40 lysis buffer (20 mM Tris/HCl pH 7.2, 200 mM NaCl, 1% NP40) supplemented with Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktails I and II (all from Sigma-Aldrich). The concentration of lysates was determined by the Bradford assay (Bio-Rad Laboratories, Richmond, CA) and 30 µg amounts of proteins were used for SDS-PAGE. Samples were analyzed by standard immunoblot procedure and visualized by chemiluminescence (Super Signal West Pico Pierce, Rockford, IL). Anti-tubulin antibody was purchased from Sigma-Aldrich Inc. (Saint Louis, MO).

Cell Growth. To determine the pro-tumor activity of Epo and SCF on breast cancer, 20.000 differentiated breast cancer cells/well were seated in the presence and in the absence of 3 U/ml Hr-Epo and 100 ng/ml of HrSCF. Both factors were added every 48 h and for three weeks in the medium. Cell growth was evaluated by cell counting every week. Human recombinant Erythropoietin (Hr-Epo) and Human recombinant Stem Cell Factor (Hr-SCF) were purchased from Peprotech Inc. (Rocky Hill, NJ).

Detection of Apoptosis. 70% confluent MCF-7 cells were shifted in PRF-SFM for 24 h. Cells were counted and 5000 cell/well were cultured for additional 24 h in the presence or the absence of Epo 3U/ml. 5-FluoroUracil (5-FU, 25 µg/ml), Methotrexate (MTX, 10 µM) and Cyclophosphamide (CPA,500 µg/ml) were subsequently added for 48h. 10.000 primary human differentiated and

undifferentiated breast cancer cells, obtained from four different patients, pre-cultured with or without Epo 3 U/ml and SCF 100 gn/ml for 24 h were exposed for 48h to Cisplatin (Cis, 3mg/ml), Doxorubicin (Doxo, 5mM), Vincristine (VCR, 1μM), Methotrexate (MTX, 10 μM) or Paclitaxel (150 ng/ml). Primary erythroid progenitor cells were seeded in a 96 well plate (10000 cells/well) and treated for 36 hours with 200 ng/ml recombinant TRAIL (LZ-TRAIL, kindly provided by Dr. Hening Walczak, Heidelberg, Germany) or Fas ligand (FasL) in the presence or absence of 100 ng/ml recombinant SCF added two hours before the treatment.. cell death was evaluated by Cell Titer 96 assay (Promega, Madison, WI). Colorimetric assay was analyzed by Victor 2 plate reader (Wallac, Turku, Finland). All chemotherapeutic drugs were purchased from Sigma-Aldrich Inc. (Saint Louis, MO).

Adult peripheral blood human progenitor cell (HPC) purification and culture. Adult peripheral blood was obtained from male donors after their informed consent and approval by the institutional Committee for Human Studies. Human $CD34^+$ precursor cells were purified from peripheral blood by positive selection using the midi-MACS immunomagnetic separation system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. $CD34^+$ cells were cultured in serum-free medium prepared as follows: Iscove's modified Dulbecco's medium (Euroclone, West York, UK) was supplemented with delipidated bovine serum albumin (10 mg/ml), pure human transferrin (0.7 mg/ml), human low-density lipoprotein (40 μg/ml), insulin (10 μg/ml), sodium pyruvate (10^{-4} mol/L), L-glutamine (2×10^{-3} mol/L), rare inorganic elements supplemented with iron sulphate (4×10^{-8} mol/L) and nucleosides (10 μg/ml each).

Mice. Four weeks old C57Bl/6 female mice weighing approximately 20g were purchased from Jackson Laboratory (Bar Harbor, ME). All were maintained with food and water ad libitum for the duration of the studies. Typically, experimental groups consisted of five mice each. Mice were observed daily and animals appearing moribund were humanely destroyed.

Stem Cells Factor and Cytotoxic Treatment. Recombinant murine SCF (Peprotech Inc., Rocky Hill, NJ) diluted in phosphate buffer saline (PBS) was injected subcutaneously at 50μg/Kg four hours before and four hours after the chemotherapeutic drugs on day 1. From day two to day 4 or 7, SCF was injected subcutaneously twice a day every 8 h. Control group received only vehicle (PBS) injection, twice a day, beginning with SCF. 5 mg/kg of Cisplatin (Teva Pharma BV, Mijdrecht, Olanda) and 100 mg/kg 5-FluoroUracile (Teva Pharma BV, Mijdrecht, Olanda) were administrated in a single intraperitoneal injection at day 1. After 4 or 7 days, blood (200 μl) was withdrawn from

the retroorbital plexum. Blood was dripped directly after removal into tubes containing 0.5M of EDTA. Analysis of peripheral blood parameter was conducted by a contract laboratory (AppiaLab, Rome, Italy) within two hours from bleeding.

After blood sampling, mice were humanely killed, femora were removed, fixed in buffered paraformaldehyde 10% for 24 h, washed and exposed to decalcificating solution (EDTA 0.05M, NaOH 5 N). For *in situ* apoptosis detection , terminal deoxy-nucleotidyl transferase-mediated dUTP nick and labelling (TUNEL) reaction was performed on 6 μm-thick paraffin-embedded sections. Briefly, sample were deparaffinised and hydrated, I*n situ* Cell Death Detection AP kit (Boehringer Mannheim, Indianapolis, IN) was used according to manufacturer's instructions.

To evaluate bone marrow cellularity, histological sections were stained with Hematoxylin/Eosin.

To evaluate single bone marrow population, from mice sacrificed on day 7, femora were harvested and marrow flushed with a 23G (0.45x10mm) syringe needle to collected single cell suspensions.

Cells were spun on a glass slide and bone marrow population evaluated by May-Grünwald-Giemsa staining and cytologic analysis. Stained cells were observed through a Nikon Eclipse E1000 transmitted light right microscope equipped with PlanFluor 40X dry and oil objectives (numerical aperture 0.75 and 1.3 respectively) and with PlanApo 60X and 100X oil objectives (numerical aperture 1.4 both). All objectives were from Nikon (Melville, NY).

Statistical Analysis. Statistical Analysis was carried out using Student's t-test.

Results

c-Kit and EpoR expression in solid tumors: The risks related to the use of hematopoietic cytokines in the supportive care of cancer patients must be carefully evaluated at the light of the recent discovery of cancer stem cells. Therefore, to determine the possible oncogenic effect of SCF and Epo, we first analyzed c-kit and EpoR expression on several differentiated and undifferentiated solid tumor populations, including ovary, lung, colon, kidney, thyroid, carcinomas and breast cancer.

We found that most cancer cells, undifferentiated and differentiated, were negative for c-kit expression, whereas EpoR positivity was found among, ovary, lung ,colon and kidney cancer (Table 1 A and B).

A

EpoR expression in cancer :

Cancer Type	Undifferentiated	Differentiated	Cell Lines
Ovary	+	NC	+++
Lung	+++	+	+++
Colon	+++	+	++
Kidney	++	+++	NC
Thyroid	-	-	NC

B

C-kit expression in cancer :

Cancer Type	Undifferentiated	Differentiated	Cell Lines
Ovary	NC	NC	+
Lung	++	+	+
Colon	-	-	+
Kidney	+	+	NC
Thyroid	-	-	NC

Table 1. Erythropoietin Receptor and c-Kit Expression in Solid Tumors Cytofluorimetric analysis of EpoR (A) and c-kit (B) expression in stem cell clones and differentiated cells obtained from human tumors of different origin. Tumor stem cells were obtained as described in Material and Methods and validated by the ability to reproduce the tumor of origin in immunodeficient mice. Differentiated cells were directly derived from surgical specimens As control immortalized human tumor cell lines were used. NC: Not Classified.

As shown in Fig. 1, breast cancer cells, undifferentiated (mammopheres) and differentiated, express EpoR, but do not express c-Kit (Fig. 2), indicating that SCF can not have a pro-tumor effect on breast cancer. Both receptors were absent in thyroid cancer cells, while c-kit was readily detectable in the TF-1 cell line used as positive control (Fig. 2).

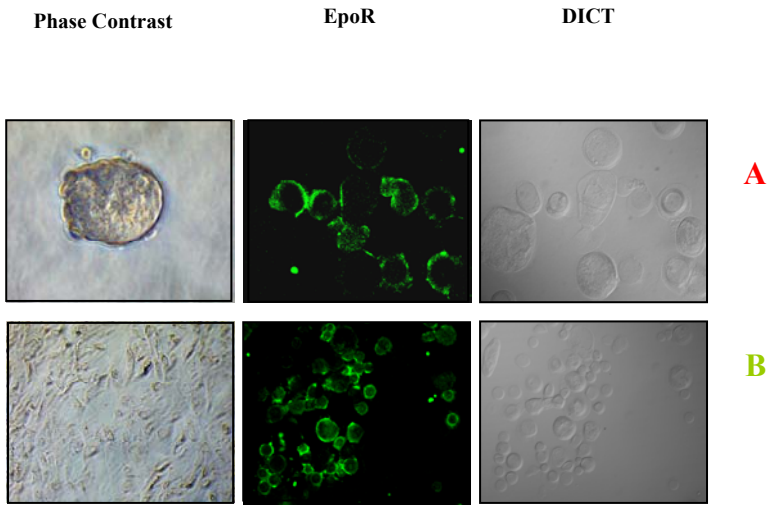

Figure 1. Erythropoietin Receptor Expression in Breast cancer Confocal microscopy analysis of breast cancer undifferentiated (A) and differentiated cells (B). After taking phase contrast images cells were fixed, cyto-spinned on glass slides for immunofluorescence microscopy and visualized with 40x objective lens. DICT states for Differential Interference Contrast with Transmitted Light.

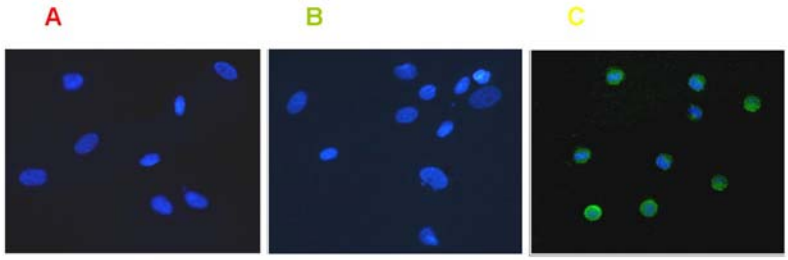

Figure 2. C-Kit expression in Breast Cancer c-kit on primary undifferentiated (A) and differentiated (B) breast cancer cells as evaluated by immunofluorescence and microscopic analysis. The hematopoietic cell line TF-1 (C) was used as positive control for c-Kit. Nuclei are in blue. One representative of five independent experiments with cells from different patients is shown.

Erythropoietin activates EpoR signalling and inhibits chemotherapy-induced apoptosis in MCF-7 breast cancer cell line. Next step was to evaluate if Epo was able to counteract the cytotoxic effects of antineoplastic drugs and eventually understand which was the molecular mechanism responsible. First we examined the influence of exposure to a pharmacologically relevant concentration of recombinant human Epo (3U/ml) on cell signalling of MCF-7 breast cancer cell line.

In MCF-7 cells , Epo was able to increase the expression of its receptor (Fig.3 A) and NF-kB, to induce activation of AKT/PKB and MAPKs and finally increase the expression of the antiapoptotic protein Bcl-XL(Fig.3 B).

Further we observed that in the presence of antitumor agent such as 5-FluoroUracil, Methotrexate and Cyclophosphamide, Epo was able to protect MCF-7 cells from drug-induced cell death (Fig.4)

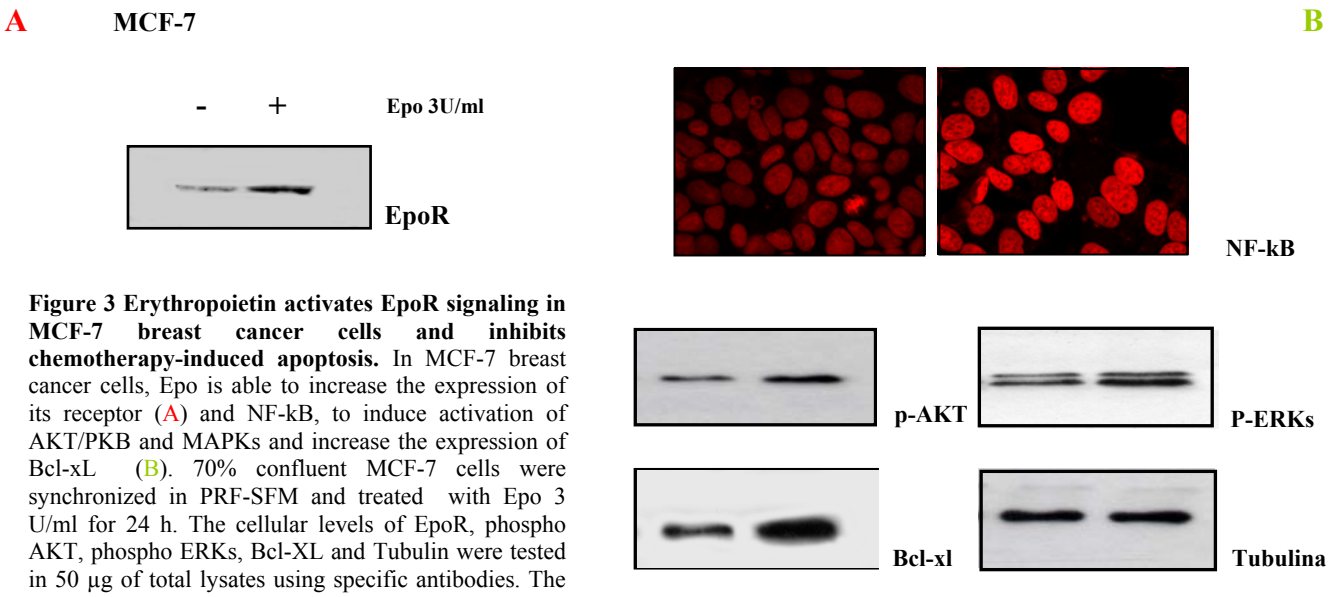

Figure 3 Erythropoietin activates EpoR signaling in MCF-7 breast cancer cells and inhibits chemotherapy-induced apoptosis. In MCF-7 breast cancer cells, Epo is able to increase the expression of its receptor (A) and NF-kB, to induce activation of AKT/PKB and MAPKs and increase the expression of Bcl-xL (B). 70% confluent MCF-7 cells were synchronized in PRF-SFM and treated with Epo 3 U/ml for 24 h. The cellular levels of EpoR, phospho AKT, phospho ERKs, Bcl-XL and Tubulin were tested in 50 µg of total lysates using specific antibodies. The expression of NF.kB was evaluated through confocal analysis using a specific immunofluorescence antibody as described in Material and Methods

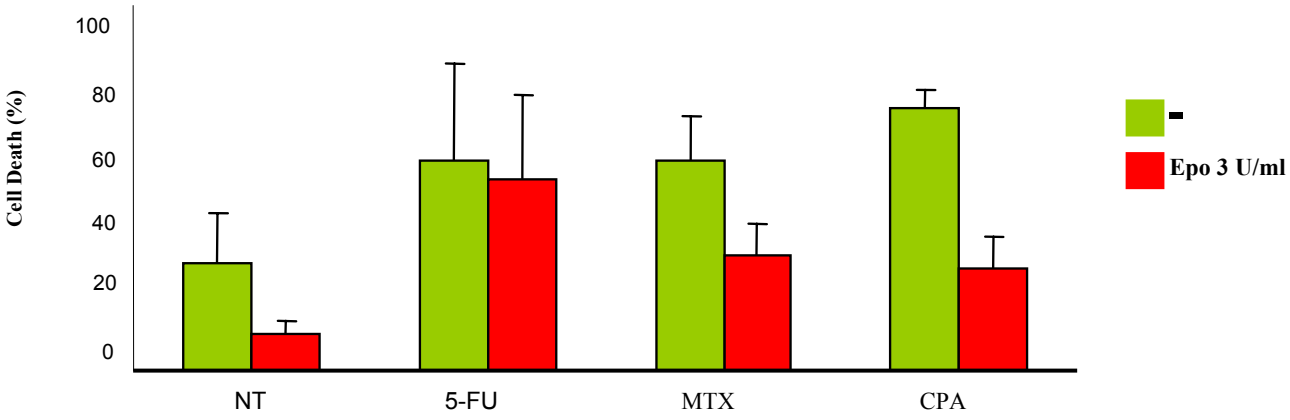

Figure 4 In breast cancer cell line Erythropoietin inhibits chemotherapy-induced apoptosis MCF-7 cells, previously synchronized in PRF-SFM were cultured with or without Epo 3U/ml for 24 h and subsequently exposed to chemotherapeutic drugs, such as 5-FluoroUracil (5-FU, 25 µg/ml), Methotrexate (MTX, 10 µM) and Cyclophosphamide (CPA,500 µg/ml) for 48h. Cell Death was evaluated by Cell Titer 96 assay as described in Material and Methods.The results are the mean ± SD of three independent experiments.

Epo increases the expansion and protects both primary differentiated and undifferentiated breast cancer cells from drug-induced cell death. In line with the absent c-kit expression in breast cancer cells, we did not observe any significant proliferative effect of SCF on primary breast cancer cultures (Fig. 5), nor the SCF interfered with chemotherapy-induced toxicity in both differentiated and indifferentiated cells (Fig.6 A and B). In contrast, the presence of Epo in the culture medium significantly increased breast cancer cell proliferation (Fig. 5) while inducing substantial cell death protection (Fig.6 A and B). These data clearly indicate that, differently from Epo, SCF is devoid of any pro-tumor effect in breast cancer cells.

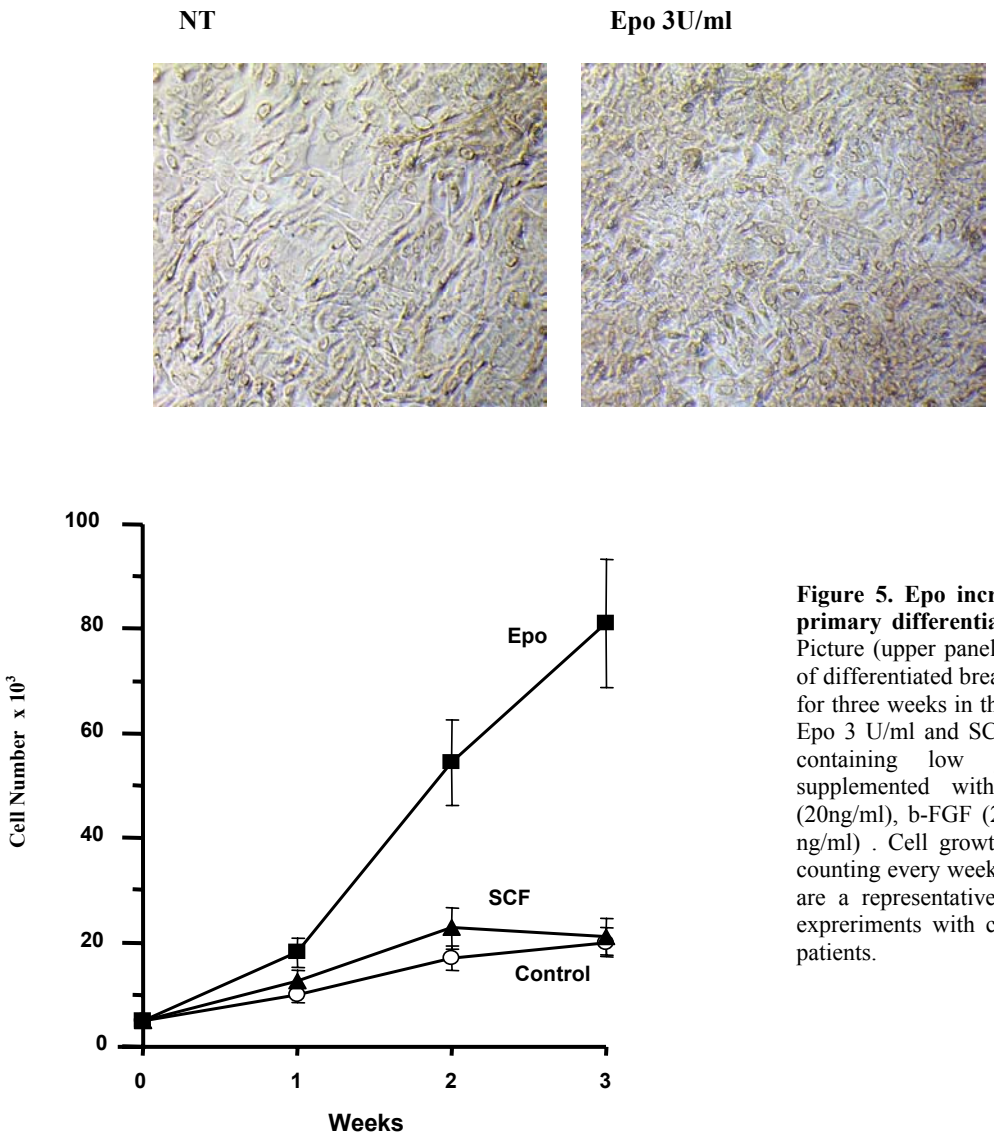

Figure 5. Epo increases the expansion of primary differentiated breast cancer cells Picture (upper panel) and cell growth curve of differentiated breast cancer cells cultivated for three weeks in the presence or absence of Epo 3 U/ml and SCF 100 ng/ml in medium containing low percentage of serum supplemented with low doses of EGF (20ng/ml), b-FGF (20 ng/ml) and Insulin (5 ng/ml) . Cell growth was evaluated by cell counting every week as indicated. The results are a representative of similar independent expreriments with cells from three different patients.

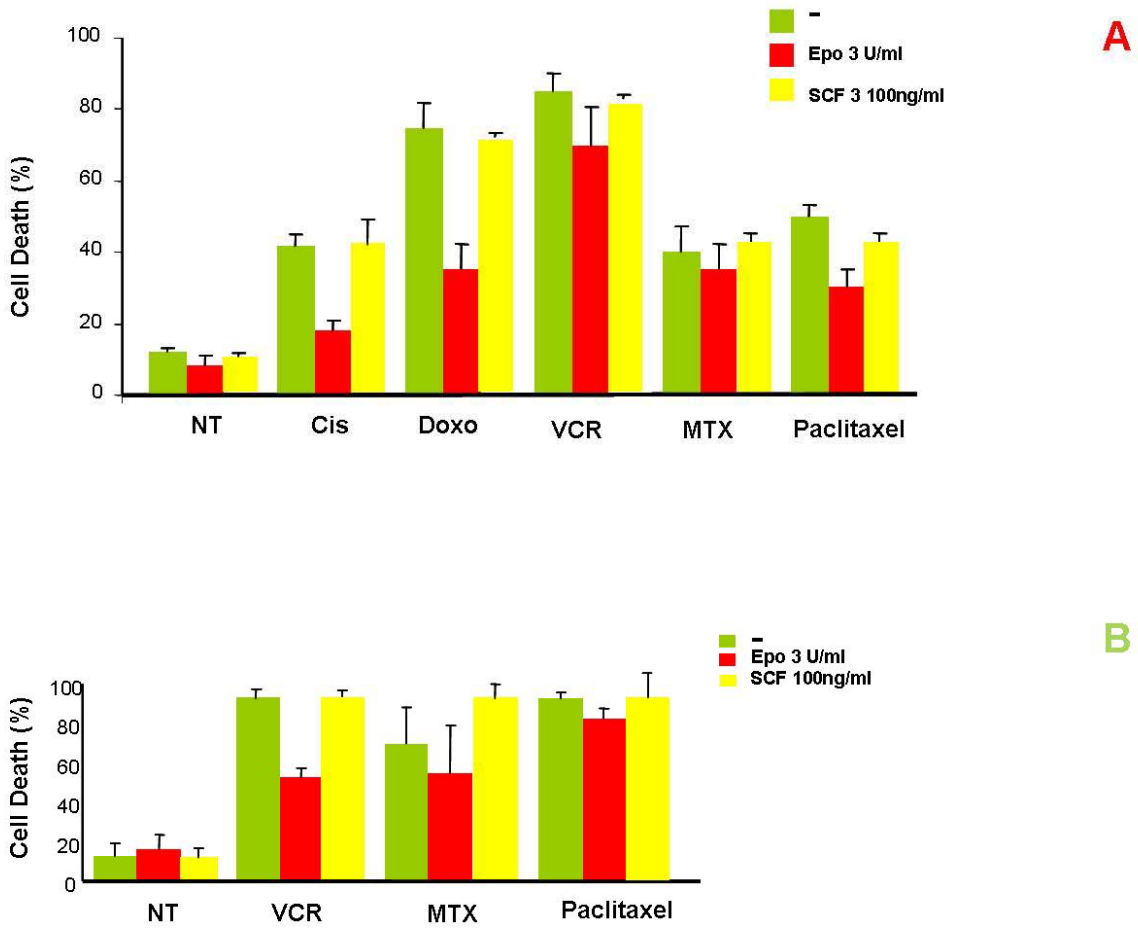

Figure 6. Epo protects cancer primary differentiated breast canecr cells and mammospheres from drug-induced cell death Primary human differentiated (A) and undifferentiated (B) breast cancer cells pre-cultured with or without Epo 3 U/ml for 24 h and then exposed for 48h to Cisplatin (Cis, 3mg/ml), Doxorubicin (Doxo, 5mM), Vincristine (VCR, 1µM), Methotrexate (MTX, 10 µM) or Paclitaxel (150 ng/ml). Cell death was determined by both MTS assay and trypan blue exclusion. The results are the mean ± SD of three independent experiments with differentiated breast cancer cells obtained from 3 different patients.

Finally, as showed in Figure 7, the presence of Epo in the culture medium was unequivocally increasing the expression of Bcl-XL, indicating this as the molecular mechanism responsible of the cytoprotective effect exert by Epo during chemotherapy treatment .

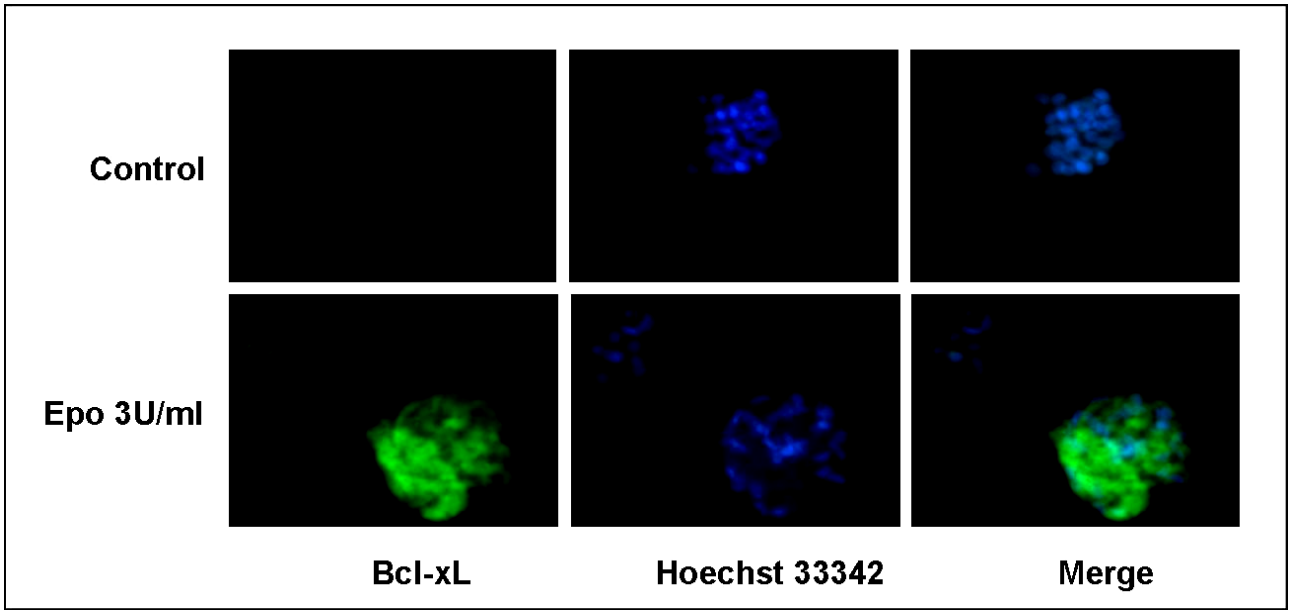

Figure 7. **Epo protects cancer mammospheres from drug-induced cell death thought increased Bcl-xL expression** Confocal analysis of Bcl-xL in undifferentiated breast cancer cells. As described in material and Methods, cells were exposed to Epo 3U/ml for 24 h, fixed and cyto-spinned on glass slides for immunofluorescence microscopy and visualized with 100x objective lens.

Stem Cell Factor protects the hematopoietic system from drug induced cell death. The c-kit receptor can be expressed by both normal and neoplastic tissues, where its stimulation by the natural ligand SCF can generate proliferative and survival stimuli. Our results showed clearly that tumorigenic breast cancer cells do not express c-kit therefore SCF can not protect them from cytotoxic effects of antineoplastic drugs. Next, considering that drug-induced apoptosis of immature (c-kit-expressing) hematopoietic cells is the primary cause of hematopoietic cell depletion in cancer patients undergoing chemotherapy (Zeuner A. et al, Blood 2003 and manuscript in preparation), we wanted to test the ability of SCF to act as a hemoprotective factor *in vitro* and *in vivo* in the presence of antitumoral agents.

In *in vitro* experiments performed on primary human erythroid progenitors we found that SCF is able to prevent apoptosis of erythroid progenitors induced by CD95/Fas ligand and TRAIL (Fig.8), one of the most promising new anticancer agent now entering phase II clinical trials, which is selectively able to induce apoptosis in tumor cells while sparing the large majority of normal cells.

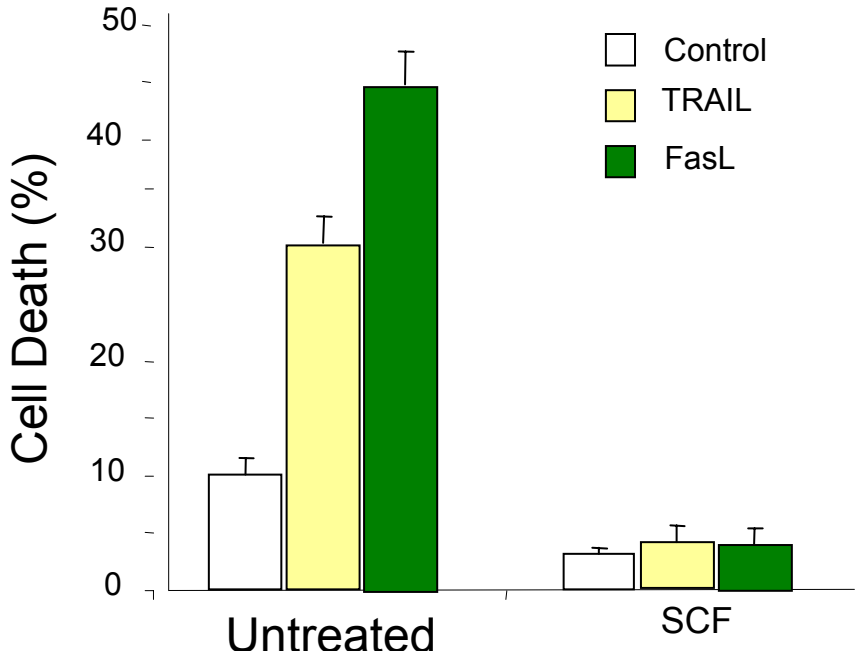

Figure 8. SCF prevents primary human erythroid progenitors cell death Percentage of cell death of primary erythroid cells treated for 36 hours with 200 ng/ml recombinant TRAIL or Fas ligand (FasL) in the presence (SCF) or absence (untreated) of 100 ng/ml recombinant SCF added two hours before the treatment.

Next we implemented the evidence that SCF can protect the hematopoietic system from chemotherapy-induced damage by analysing the effects of SCF *in vivo* in experimental models of chemotherapy-induced cytotoxicity. C57BL/6 mice were subdivided in groups of four and treated with saline solution (PBS), with Cisplatin or 5-FluoroUracile (injected intraperitoneally), with SCF alone (injected subcutaneously) and with cisplatin + SCF or 5-FluoroUracile + SCF.

SCF was administered two hours before the chemotherapeutic agent and every day for the following 4 days and 7 days. Blood cell values were assessed 4 and 7 days after chemotherapic administration and representative results are shown in Table 2.

Group	RBC	Hb	WBC	PLT
Control (PBS)	9.11 x 10^6 /mm^3	14.4 g/100 ml	15.4 x 10^3 /mm^3	850 x 10^3 /mm^3
Cisplatin	6.95 x 10^6 /mm^3	9.7 g/100 ml	10.9 x 10^3 /mm^3	515 x 10^3 /mm^3
SCF (SC)	9.25 x 10^6 /mm^3	14.2 g/100 ml	15.8 x 10^3 /mm^3	903 x 10^3 /mm^3
SCF(SC) + Cis	8.75 x 10^6 /mm^3	13.7 g/100 ml	13.3 x 10^3 /mm^3	860 x 10^3 /mm^3

Table 2. Blood values of mice treated with saline solution (PBS), with cisplatin (5 mg/kg of weight), with SCF (100 micrograms/kg/day), injected subcutaneously (SC) or with SCF + cisplatin (SCF + Cis). Values refer to a retro-orbital bleeding done 7 days after cisplatin administration. RBC = red blood cells, Hb = haemoglobin, WBC = white blood cells, PLT = platelets. . The results are a representative of 5 independent expreriments .

In a different set of experiments, mice treated with Cisplatin or 5-Fluorouracil (5-FU) in the presence or in the absence of SCF were sacrificed 4 and 7 days after the chemotherapic insult to obtain femoral bone marrow sections. Histological sections for TUNEL assay show in Fig. 9 presence of apopototic cells in mice treated with only Cisplatin while Stem Cell Factor in mice treated with both was able to prevent cell depletion.

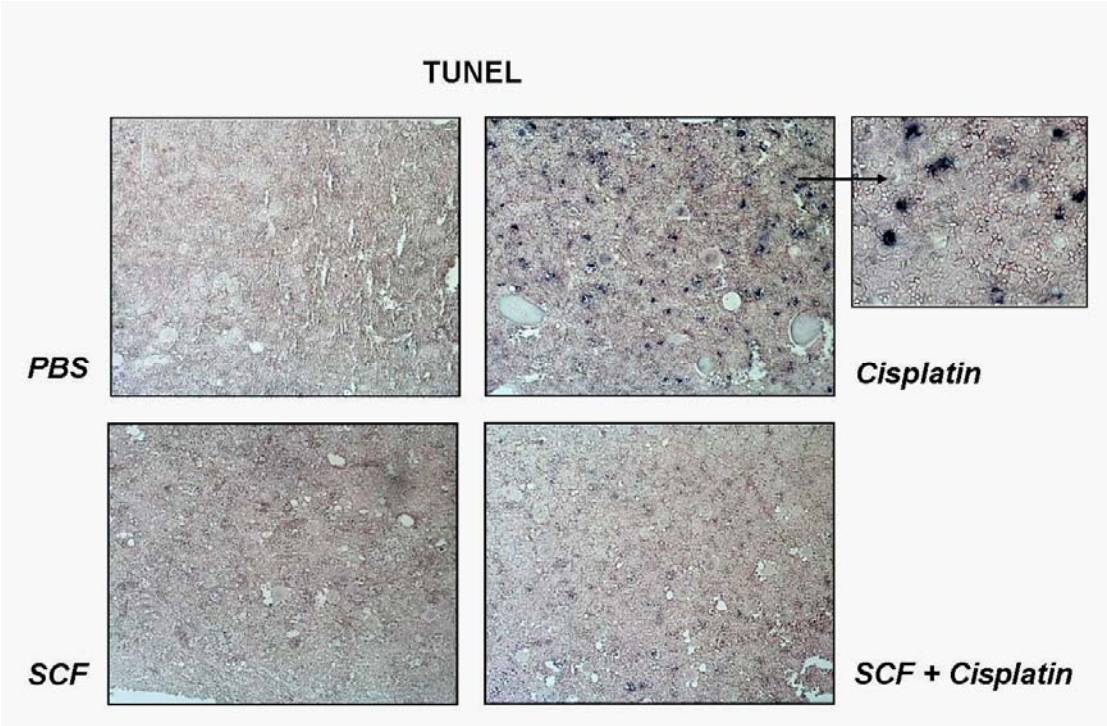

Figure 9. SCF prevents Apoptosis in vivo. Tunel Assay on bone marrow histological sections of mice treated with PBS, Cisplatin (5 mg/Kg), SCF (100 µg/Kg/day) and the combination of both for 4 days. Chemotherapeutic agents was administered on day one intraperitoneally, whereas SCF was injected subcutaneously twice a day, from day 1 to day 4 as described in Material and Methods.

In the second set of experiment mice treated with cisplatin or 5-FU display strong marrow hypoplasia with myelofibrosis and clusters of megakaryocytes. Treatment of mice with SCF consecutively for a week resulted, in both cases, in a strong myeloprotection, as shown by high bone marrow cellularity and the almost complete disappearance of dysplastic cells (Fig.10 A). The relative percentages of bone marrow cellularity are shown in Fig.10 B.

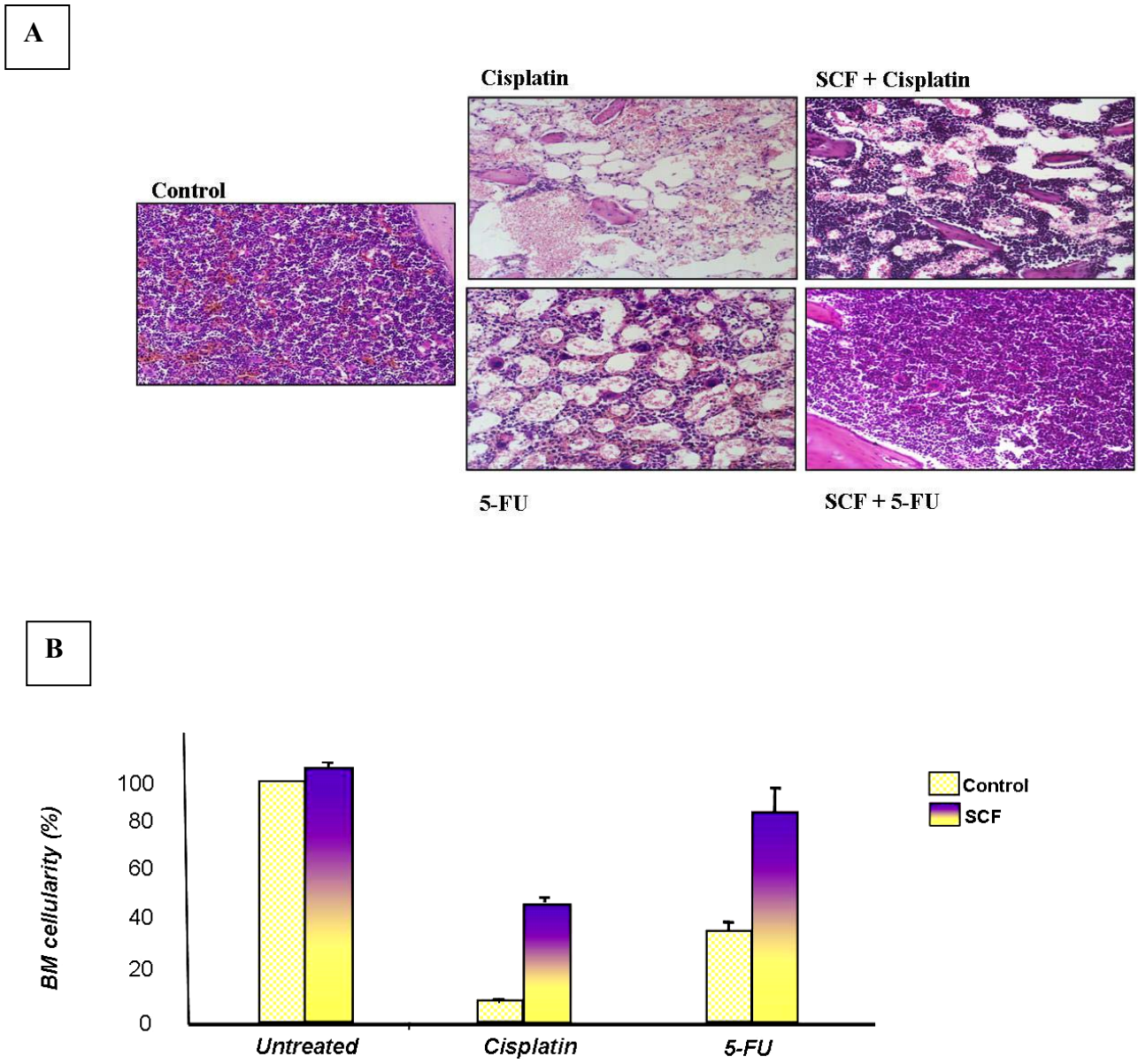

Figure 10. **Bone Marrow cellularity in C57Bl6 mices**. **A)** Bone marrow histological sections stained with Hematoxylin/Eosin obtained from mice injected intraperitoneally with PBS (Control), with cisplatin (Cis, 5 mg/kg of weight) or with 5-Fluorouracil (5-FU, 100 mg/kg), with or without SCF (100 micrograms/kg/day). Chemotherapeutic agents were administered on day1 whereas SCF was given twice a day, from day 1 to day 7 as described in Material and Methods. **B**) Bone marrow cellularity of samples treated with chemotherapeutic agents with or without SCF as above. Panel B shows the means and standard deviations of results obtained from three independent experiments with chemotherapy.

In our expreriments, we also analyze the entire bone marrow population, and as shown in Figure 11 we observe an increase in all bone marrow cells of mice treated with SCF and chemotherapeutic agents compared to those treated with the drug alone.

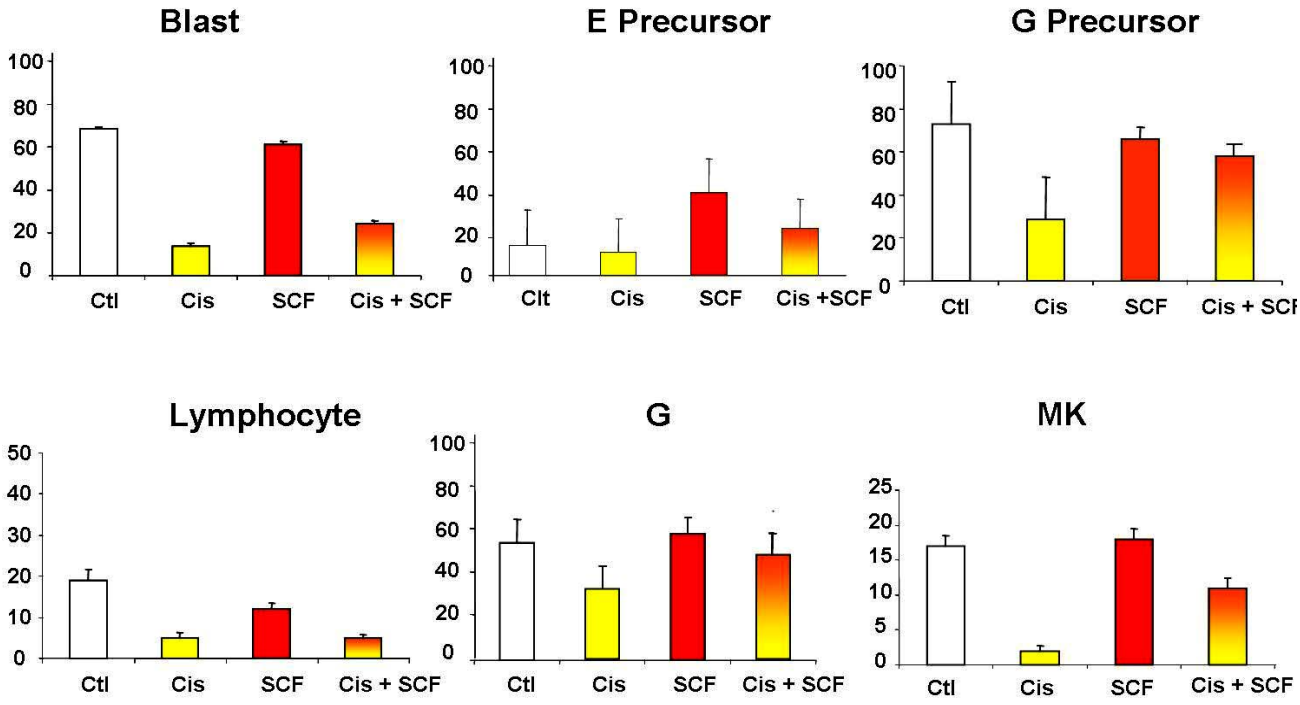

Figure 11. SCF acts an hemoprotective agent on the entire bone marrow population. Chemotherapeutic agent was administered on day 1 whereas SCF was given twice a day, from day 1 to day 7 as described in Material and Methods. After a week, mices were sacrified and and bone marrow from the femurus flashed to obtained cell population. Cells were washed, counted and spun on a glass slide for May-Grünwald- Giemsa- staining. Results are the mean of three separate experiments (each group represents the data from 15 anumals).

The experiments described above demonstrate that SCF protects the hematopoietic system from chemotherapy-induced damage *in vivo* and outline a protocol for a potential clinical application of SCF to prevent chemotherapy-induced cytotoxicity, thus considerably reducing the amount of experimentation needed for a future use of SCF in cancer patients.

Discussion

Anaemia is a common symptom associated with most cancer patients, and appears in all patients with hematological malignancies [4].In a recent European survey evaluating anaemia in over 13,000 patients with malignancies, anaemia was observed in 68% of patients at some time during the 6-month survey [45]. The manifestations of anaemia, including fatigue, dizziness, headache, shortness of breath, chest pain and depression, impact on the overall quality of life (QoL) of anaemic patients with cancer.

The ability of erythropoietin (Epo) to promote the production of red cells is currently exploited to treat chemotherapy-induced anemia. Epo significantly increases haemoglobin (Hb) levels, reduces transfusion requirements, and improves quality of life, particularly by relieving fatigue[4] .

However, recent studies have shown that Epo has biological functions aside from regulating erythropoiesis and that many solid tumors express EpoR, raising concerns about the fact that Epo could stimulate the growth of indolent tumors and interfere with the action of antitumor therapies, either by enhancing tumor proliferation rates or interfering with apoptotic cell death, therefore the expression of Epo receptor (EpoR) in a variety of cancer cells suggests that Epo-based supportive therapy can negatively affect the clinical outcome.

Recently, the presence of c-kit, the receptor of an other essential factor for erythropoiesis, the Stem Cell Factor (SCF), has been extensively investigated in cancers of various origin as this molecule represents a target for the tyrosine kinase inhibitor imatinib mesylate (Gleevec), thus rendering available an unprecedented array of data on c-kit expression in solid and haematological tumors.

In a comprehensive investigation [1,15], 81/120 tumor types examined did not yield any sample positive for c-kit expression. C-kit negative tumors included both solid and hematologic tumors, suggesting that the use of SCF should be safe in many of the most common malignancies.

More over, the risks related to the use of hematopoietic cytokines in the supportive care of cancer patients must also be carefully evaluated at the light of the recent discovery of cancer stem cells. The existence of a undifferentiated subpopulation of tumorigenic cells responsible for tumor maintenance, growth and spreading was known since several years in leukemias, but has been now demonstrated to occur also in solid tumors such as breast and brain cancers [19, 20].

The possibility that cancer stem cells may use hematopoietic growth factors to proliferate and resist to apoptotic stimuli poses an additional caveat for the administration of cytokines to cancer patients and requires a careful assessment of the presence of cytokine receptors on the surface of both stem and differentiated cancer cells.

In our laboratory, we have obtained cancer stem and differentiated cells from several solid tumors including breast, thyroid, colon, and lung carcinomas etc.

We analysed the expression of EpoR and c-kit in a variety of cancer types and we found that the large majority of stem and differentiated cancer cells tested resulted negative for c-kit expression and positive for EpoR expression. In breast cancer EpoR and c-kit have a different expression patterns. Ulivi et al [77] demonstrated that c-kit expression is downregulated toward a more aggressive phenotype, while EpoR expression is upregulated .

The aim of this study was to evaluate the possible oncogenic effect of SCF on differentiated and undifferentiated breast cancer cells and eventually outline a protocol for a potential clinical application of SCF to prevent chemotherapy-induced anemia in this type of cancer. Our data showed that breast cancer cells, undifferentiated and differentiated, express EpoR, but do not express c-Kit, indicating that SCF can not have a pro-tumor effect on breast cancer**.** In line with the absent c-kit expression in breast cancer cells, we did not observe any significant proliferative effect of SCF on primary breast cancer cultures, nor the SCF interfered with chemotherapy-induced toxicity in both differentiated and undifferentiated cells.

In contrast, the presence of Epo in the culture medium significantly increased breast cancer cell proliferation while inducing substantial cell death protection. The presence of Epo in the culture medium was unequivocally increasing the expression of the antiapoptotic protein Bcl-XL, indicating this as the molecular mechanism responsible of the cytoprotective effect exert by Epo during chemotherapy treatment. These data clearly indicate that, differently from Epo, SCF is devoid of any pro-tumor effect in breast cancer cells.

In clinic, drug-induced apoptosis of immature hematopoietic cells is the primary cause of hematopoietic cell depletion in cancer patients undergoing chemotherapy, more over mobilization of erythroid progenitors population is a major issue in cancer related anemia .

Horsfall et al. [52] have demonstrated that the combination of G-CSF and SCF mobilizes the highest number of progenitor cells and some clinical trials in humans with multiple myeloma, breast cancer and lymphoma SCF has been used successfully to mobilize stem and progenitor cells [17, 18] . In some cases, co-administration of G-CSF and SCF compared to G-CSF alone in breast cancer patients has been shown to result in reduced level of tumor cell contamination and *in vitro* introduction of c-kit into a breast cancer cell line MCF-7 mediate inhibitory signals for the growth of breast cancer cells[51]. In the second part of our studies, we wanted to test the ability of SCF to act as a hemoprotective factor *in vitro* and *in vivo* in the presence of antitumoral agents.

We implemented the evidence that SCF can protect the hematopoietic system from chemotherapy-induced damage in *in vitro* experiments performed on primary human erythroid progenitors, SCF was able to prevent apoptosis of erythroid progenitors induced by CD95/Fas ligand and TRAIL.

In *in vivo* in experimental models of chemotherapy-induced cytotoxicity we analyze the entire bone marrow population, and we observe an increase in all bone marrow cells of mice treated with SCF and chemotherapeutic agents compared to those treated with the drug alone. Treatment of mice with SCF resulted in a strong myeloprotection as demonstrated by TUNEL Assay.

Analysis of peripheral blood values showed and increase in cells number after stem cell factor administration, indicating the ability of SCF to restore a normal erythropoiesis.

In summary, we clearly showed that SCF effectively protects normal cells from cytotoxic stimuli both *in vitro* and *in vivo.* Moreover, we provide clear evidence that many tumors do not express the c-kit receptor, even at cancer stem cell level, particularly in breast cancer, the Stem Cell Factor does not have any pro-tumor activity, as a further confirmation of the safety of SCF administration and outline a protocol for a potential clinical application of SCF to prevent chemotherapy-induced cytotoxicity, thus considerably reducing the amount of experimentation needed for a future use of SCF in cancer patients.

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Role of Estrogen Receptor α in Modulating IGF-I Receptor Signaling and Function in Breast Cancer

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The insulin-like growth factor I (IGF-I) receptor (IGF-IR) is a multifunctional transmembrane tyrosine kinase that has been implicated in neoplastic transformation. The tumorigenic potential of IGF-IR relies on its strong anti-apoptotic and mitogenic activity. The growth and survival signals of IGF-IR are mediated through multiple intracellular pathways, many of which emanate from insulin receptor substrate 1 (IRS-1). In hormone-dependent breast cancer cells, IGF-IR and IRS-1 are often co-expressed with the estrogen receptor α (ERα), and IGF-I and ER systems are engaged in a powerful functional cross-talk. Most notably, activation of ERα upregulates the expression of IRS-1, IGF-IR, and IGF-1, which results in amplification of IGF-I responses. Reciprocally, stimulation of IGF-IR increases the phosphorylation and activity of ERα.
In contrast, in ERα-negative breast cancer cells and tumors, the levels of IGF-IR and IRS-1 are often decreased and IGF-I is non-mitogenic. Our data suggest that defective IGF-IR signaling in ERα-negative cells is related, at least in part, to improper activation of the IRS-1/PI-3K/Akt/GSK-3 pathway and lack of Rb1 phosphorylation. These defects are partially reversed by re-expression of ERα. Interestingly, some non-mitogenic IGF-I responses, such as migration and invasion are retained in the absence of ERα, suggesting that IGF-IR function in breast cancer cells might depend on the ERα status. The understanding of how ERα may dictate IGF-I responses will help in devising rational anti-IGF-IR strategies for breast cancer treatment.

Key Words: IGF-I, Estrogen receptor, Breast cancer, Apoptosis, Migration, Invasion

Although the insulin-like growth factor-I receptor (IGF-IR) regulates normal cell growth, its abnormal activation has been linked with the development of numerous neoplastic diseases, including hormone-dependent cancers (1-11).

The ongoing efforts of the pharmaceutical industry will likely result in the development of anti-IGF-IR drugs. In fact, several compounds targeting IGF-IR and/or its signaling molecules, such as anti-IGF-IR antibodies and small molecule inhibitors targeting IGF-IR tyrosine kinase are already being tested in preclinical trials (12, 13). The rational use of such compounds in the therapy of hormone-dependent breast cancer will require thorough understanding of the interactions between the IGF-IR system and the steroid hormone system. Here, we review IGF-IR activities and address differences in IGF-IR signaling and function in ERα-positive and ERα-negative breast cancer cells.

Activities and signaling of IGF-IR

IGF-IR is an evolutionary conserved and ubiquitous transmembrane tyrosine kinase activated by IGF-I, IGF-II or insulin at hyperphysiological doses (14). As many other "ancient" receptors, IGF-IR has the ability to regulate diverse biological processes, for instance proliferation, survival, transformation, differentiation, cell-cell and cell-substrate interactions (8, 12-21). IGF-IR signal specificity in a given cellular background is dictated by the engagement of different sets of intracellular pathways. The activation of specific pathways and induction of specific functions depends on the number of activated receptors, availability of intracellular signal transducers and action of negative regulators, e.g., phosphatases (8). Studies on mouse fibroblasts and breast cancer cells suggested that low IGF-IR levels (~10^3 receptors/cell) are sufficient to promote survival and migration, but are not able to sustain mito-

genesis. Higher expression of IGF-IR (~10^4 receptors/cell) appeared to induce mitosis, but provided only weak transforming activity as measured by soft agar growth, while activation of more than $1x10^5$ IGF-IRs/cell promoted robust transformation (22,23,24). Similar studies with animal models confirmed direct relationship between the number of stimulated IGF-IRs and cell survival and/or tumorigenesis (25, 26).

In addition to the cellular context, the extent of IGF-IR activation depends on extracellular factors, especially on the abundance of bioavailable ligands. The abundance of free IGF-I and IGF-II is regulated by several IGF binding proteins (27). It has been postulated that elevated levels of bioavailable endocrine IGF-I as well as locally produced IGF-I, IGF-II, or insulin could hyperactivate IGF-IR, increasing its tumorigenic activity (5,11,28). Indeed, studies in animals clearly indicated that high doses (4-10 mg/kg) of systemic IGF-I can reduce the latency of tumor development (25). Furthermore, some recent reports suggested that higher plasma concentrations of IGF-I are linked with an increased risk of several common neoplasms, i.e., cancers of the breast, prostate, lung, and colon (5). It is possible that higher plasma levels of IGF-I reflect its paracrine or autocrine synthesis within tissues other than the liver (29). In this context, it is worth noting that many human tumors can synthesize IGF-I and/or IGF-II that likely stimulate autonomic growth (5, 9).

Activation of IGF-IR results in tyrosine phosphorylation of its cytoplasmic β-subunit, followed by the recruitment of IGF-IR substrates, of which the most notable are insulin receptor substrate 1 (IRS-1) and src- and collagen-homology (SHC) protein. IGF-IR-induced tyrosine phosphorylation of IRS-1 and SHC allows them to bind several effector proteins (enzymes and/or adapters) and activate a variety of downstream signaling pathways (8,15,20,30-32).

A well-recognized and extensively studied function of IGF-IR is its anti-apoptotic activity (16-18,20,21,33). IGF-IR can transmit survival signals through multiple, partially redundant, pathways. For instance, there are at least 3 pathways induced by IGF-IR, all leading to the phosphorylation and inactivation of a pro-apoptotic protein BAD. One of these pathways is the classical antiapoptotic response and includes tyrosine phosphorylation of IRS-1, binding of PI-3 kinase (PI-3K) to IRS-1, and activation of PI-3K and its downstream effector Akt kinase (Akt) (31,34). Another survival signal is transmitted through the SHC/Ras/ERK1/2 pathway, and a distinct pathway depends on mitochondrial translocation of Raf-1 (35,36).

IGF-I-dependent stimulation of Akt can repress the activity of GSK-3β kinase, blocking proteasomal degradation of GSK-3β targets, e.g., Cyclin D1 (37,38). In effect, accumulation of Cyclin D1 can stabilize the cyclin D1:cdk4 complex, resulting in hyperphosphorylation and inactivation of cell cycle inhibitor Rb1, and transcriptional stimulation of growth-related genes. Another target of GSK-3β that has been reported to accumulate in IGF-I-stimulated cells is β-catenin (39-41). The accumulation of β-catenin, its translocation to the nucleus and binding to the Tcf (T-cell factor) transcriptional complex can activate transcription of several growth/survival proteins, such as c-Myc, Cyclin D1, and Id2 (42-44). Further positive effects of IGF-IR on cell cycle machinery include stimulation of cyclin E expression and increased activation of cyclin E:cdk2 complexes (23,45). Other mitogenic/survival IGF-IR pathways operating in some cellular systems involve signal transducers and activators of transcription (STATs) (46-48).

Importantly, activation of IGF-IR growth/survival pathways can be influenced by cell-matrix interactions, for instance SHC (49,50), IRS-1, and IRS-2 (51-53), associate with different integrin receptors and transmit matrix-dependent survival signals. IGF-IR has also been shown to regulate the activity of several proteins engaged in cytoskeleton reorganization, such as FAK, paxillin, p130 Cas (54,55). In addition, the elements of IGF-IR signaling system (IGF-IR, IRS-1, SHC) can interact with molecules in cell-cell junctions (19), and IGF-IR can stimulate intercellular adhesion and improve cell survival under anchorage-independent conditions (19,56-58).

IGF-IR and breast cancer development

IGF-IR plays an important role in the development of normal breast; the same receptor, however, has been implicated in the etiology of breast cancer. It is believed that in case of mammary epithelium, tumorigenic activity of IGF-IR is related to hyperactivation of its survival pathways (5-9). Indeed, in breast cancer, several components of the IGF system are deregulated in a manner promoting IGF-I responsiveness. First, IGF-IR appears to be significantly (several-fold) overexpressed and highly activated in primary breast cancer compared with its status in normal epithelial cells (59-62). Moreover, breast cancer cells have been shown to overexpress insulin receptor/IGF-IR hybrid receptors that are known to induce typical IGF-I responses (63). On the other hand, the IGF-II receptor,

which normally serves as "molecular sink" to bind and downregulate IGF-II, has been found non-functional in some breast tumors (64). IGF-IR ligands, IGF-I and IGF-II, and insulin are mitogens for many breast cancer cells *in vitro* and *in vivo*, and histological data demonstrated that breast tumors express IGFs (9). Epidemiological studies, although have not established causality, suggested a role of IGF-I system in breast cancer. Elevated expression of IGF-IR or IRS-1 in primary tumors has been linked with increased drug-and radio-resistance and cancer recurrence (65-67). In addition, several studies found that higher levels of free circulating IGF-I in combination with lower levels of IGF-I binding protein 3 correlated with moderately increased breast cancer risk in premenopausal women (5).

ER/IGF-IR cross-talk in breast cancer

Accumulating evidence suggests a strong link between the IGF-I and ER systems. In ERα-positive breast cancer cell lines, ERα and IGF-IR have been shown to be engaged in a functional cross-talk. The aspects of this cross-talk include potentiation of IGF-I responses by ERα, stimulation of ERα activity by IGF-I, and activation of common sets of intracellular pathways leading to additive or synergistic effects (8,68,69).

In the first instance, ERα sensitizes cells to IGF by upregulating several elements of IGF-I system. Specifically, 17-β-estradiol (E2) treatment stimulates the expression of IRS-1 mRNA and protein, promoting IRS-1/PI-3K/Akt signaling (8,65,70,71). ERα is also responsible for the upregulation of IGF-I mRNA expression (72). In addition, some data indicated increased expression of IGF-IR mRNA upon E2 treatment (73). The transcriptional effects of ERα appear to be specific for the IGF-I/IGF-IR/IRS-1 pathway, as E2 does not affect the expression of other IGF-IR substrates such as SHC, IRS-3, and IRS-4 (65,70,74). Consistent with these observations is the fact that antiestrogens Tamoxifen and ICI 182,780 downregulate the expression and signaling of IGF-IR and IRS-1, but have no effect on SHC (8).

In light of these data, one can speculate that overexpression of IGF-IR and/or IRS-1 that has been documented in a subset of breast tumors could impede antiestrogen therapy. Indeed, our experiments with breast cancer cells *in vitro* demonstrated that overexpression of IGF-IR or IRS-1, but not SHC, stimulates E2-independence and antiestrogen-resistance in breast cancer cells (19,75-77).

Interestingly, new data suggested that ERα is able to influence IGF-IR signaling not only on the transcriptional but also on the post-transcriptional level. Specifically, a fraction of ERα has been found in association with IGF-IR, SHC, PI-3K, or IRS-1, in all cases improving activity of these signaling molecules (79-82). Our recent observations suggest that unliganded ERα may decrease proteasomal degradation of IRS-1, leading to enhanced IRS-1 signaling in the presence of IGF-I (79).

Just as ERα upregulates IGF-I responses, IGF-I can enhance ERα effects. For example, it has been shown that IGF-I enhances transcriptional activity of ERα in a ligand-independent manner and increases expression of E2-inducible genes (83,84). The phosphorylation of ERα is known to be stimulated by several IGF-I-dependent pathways, such as the ERK1/2 and Akt pathways (85,86).

Both ERα and IGF-IR are known to upregulate breast cancer cell growth by inducing the same sets of intracellular pathways. For instance, similar to IGF-I, E2 has been shown to stimulate ERK1/2 and Akt pathways, increase accumulation of Cyclin D1 and phosphorylation of Rb1 in breast cancer cells (68,87). Consequently, co-stimulation with IGF-I and E2 has been shown to have synergistic effects on cell cycle progression (45,88).

Effects of IGF-IR in ERα-negative breast cancer cells

The results obtained with clinical material as well as studies on breast cancer cell lines suggested that IGF-IR expression and IGF-I responsiveness are linked with ER positivity (60,62,89-91). On the other hand, many ERα-negative breast cancer cell lines do not respond to IGF-I with growth and/or survival (24,92-95). In agreement with this notion, depletion of ERα in MCF-7 cells has been shown to abrogate the mitogenic response to IGF-I, while re-introduction of ERα restores IGF-I-dependent growth (96). We obtained similar results with ERα-negative MDA-MB-231 cells re-transfected with ERα, where ectopic expression of ERα improved survival in IGF-I (79).

Despite reduced IGF-I requirements for proliferation and survival, different ERα-negative breast cancer cells appear to depend on IGF-IR expression for migration and metastasis. For instance, blockade of IGF-IR in MDA-MB-231 cells by anti-IGF-IR antibody reduced migration *in vitro* and tumorigenesis *in*

Table I - IGF-I responses in ERα-positive and ERα-negative cell lines

	Cell Number in IGF-I (% over untreated)			Migrating Cells (% over SFM)
Cell Line	Day 1	Day 2	Day 4	IGF- 1
MDA-MB-231 (ER-)	- 10±2	- 25±3	- 70±10	+ 45±5
MDA-MB-436 (ER-)	+ 7±3	- 57±6	- 55±7	+ 45±10
MDA-MB-468 (ER-)	+ 5± 1	- 35±3	- 80±8	+ 47±4
BT-20 (ER-)	- 40±5	- 67±5	- 75±8	+ 72±6
T-47D (ER+)	+ 12±2	+ 89±8	+ 84±9	+ 20±5
MCF-7 (ER+)	+ 10±4	+ 180±12	+ 104±10	+ 25±2

Growth/survival. The cells were plated in 6-well plates at a concentration of 1.5-2.0x10^5 cells/plate in DMEM:F12 (1:1) containing 5% CS. The following day (day 0), the cells at approximately 70% confluence were shifted to phenol red-free serum-free medium (SFM) containing 50 ng/ml IGF-I or were left untreated. Cell number was determined at 0, 1, 2, and 4 days. The results represent the change (%) in cell number in respect to untreated control at the tested time. Note: MCF-7 and T47D cells proliferate in SFM. Cell Migration. The migration of cells was studied in modified Boyden chambers containing porous (8 μm), polycarbonate membranes. 2x10^4 cells (synchronized in SFM for 24 h) were suspended in 200 μl of SFM and plated into upper wells. Lower wells contained 500 μl of SFM or SFM plus 50 ng/ml IGF-I. After 12 h, the cells in the upper wells were removed, while the cells that migrated to the lower wells were fixed and stained in Coomassie Blue solution. The cells that migrated to the lower wells were counted under the microscope. The experiments were repeated at least three times, the results are average +/- SE.

vivo, and expression of a soluble dominant-negative IGF-IR in MDA-MB-435 cells inhibited cell-matrix adhesion and impaired metastasis in animals (24,97-99). Additionally, in a highly metastatic variant of MDA-MB-231 cell line, downregulation of IRS-2 reduced migration and invasion (100).

Table I includes our data on IGF-I responses in several breast cancer cell lines. The results support the idea that IGF-I can stimulate migration of ERα-negative breast cancer cells without producing any mitogenic effects, while the same treatment stimulates both responses in ERα-positive cells. Thus, it is possible that ERα controls IGF-I growth and survival activities, but not other IGF-I-dependent functions, such cell motility.

Differential IGF-IR signaling and function in ERα-positive and ERα-negative breast cancer cells

What are the molecular basis underlying differential IGF-I function depending on the ERα status? We addressed this point with MDA-MB-231 cells as a model. These cells are ERα-negative, express low levels of IGF-IR and moderate levels of IRS-1 (24). In MDA-MB-231 cells, IGF-I stimulates cell migration but is not mitogenic or anti-apoptotic (24). We probed whether the lack of IGF-I growth response in these cells relates simply to low IGF-IR expression. To this end, we generated, by stable transfection, MDA-MB-231 clones with high IGF-IR expression (MDA-MB-231/IGF-IR cells). In MDA-MB-231/IGF-IR cells, epigenetically expressed IGF-IRs were fully functional as measured by the extent of IGF-IR autophosphorylation and activation of IRS-1. However, IGF-I treatment did not increase MDA-MB-231/IGF-IR cell growth and survival (24). This suggested that defects in IGF-I mitogenic signaling localize downstream of IRS-1. Similar conclusions were reached by Jackson and Yee (92), who demonstrated that overexpression of IRS-1 in ERα-negative MDA-MB-468 and MDA-MB-435A cells failed to restore IGF-I mitogenic response.

The analysis of our MDA-MB-231/IGF-IR cells and comparison with ERα-positive MCF-7/IGF-IR cells expressing similar levels of IGF-IR suggested that in the absence of ERα, IGF-I activates the PI-3K/Akt pathway only transiently, while in the presence of ERα, IGF-I can induce this pathway in a sustained manner (at least 24 h) (24). Additional differences were noted in the activation of the ERK1/2 pathway: ERK1/2 kinases were constitutively activated in

Table II - Differential effects of IGF-I on the activation of signaling proteins in ERα-positive and ERα-negative breast cancer cells

PROTEIN	MCF-7		MDA-MB-231		MDA-MB-231/ER	
	15 min	**1 day**	**15 min**	**1 day**	**15 min**	**1 day**
Adducin (S662)	Up (92%)	Up (400%)	Same	Same	Same	Same
S6p70 (T389)	Same	Same	N/A	N/A	Same	Same
FAK (Y861)	Same	Same	N/A	N/A	Same	Same
ERK 1 (T202/Y204)	**Same**	**Same**	**Same**	**Down (50%)**	**Same**	**Same**
ERK 2 (T189/Y187)	**Up (97%)**	**Up (60%)**	**Same**	**Same**	**Up (52%)**	**Down (50%)**
RSK 1 (T360/S364)	Same	Same	Up (50%)	Up (100%)	Same	Same
RAF 1 (S259)	Same	Same	Up (130%)	Same	Same	Same
MEK 2 (S221)	Up (220%)	Same	Down (50%)	Same	Up (90%)	Same
Akt 1 (T308)	Up (300%)	Up (85%)	Up (50%)	Down (100%)	N/A	N/A
Akt 1 (S473)	**Up (420%)**	**Up (200%)**	**Up (250%)**	**Down (100%)**	**Up (370%)**	**Up (80%)**
GSK 3α (S21)	**Up (130%)**	**Up (150%)**	**Same**	**Down (50%)**	**Up (170%)**	**Up (50%)**
GSK 3β (S9)	**Up (70%)**	**Up (50%)**	**Same**	**Down (100%)**	**Up (50%)**	**Up (50%)**
GSK 3α (Y279)	Same	Up (50%)	Down (50%)	Down (50%)	Same	Up (50%)
GSK 3β (Y216)	Same	Same	Same	Same	Up (80%)	Same
PKCε (S716)	Same	Same	Same	N/A	N/A	N/A
PKCα (S657)	Same	Same	Same	Down (70%)	Down (60%)	Same
PKC α/β (T638)	Same	Up (88%)	Same	Down (85%)	Down (50%)	Same
Src (Y529)	**Same**	**Down (50%)**	**Same**	**Same**	**Same**	**Down (50%)**
PKCδ (T505)	Same	Same	Down (2x)	N/A	N/A	N/A
MEK6 (S207)	Same	Up (130%)	N/A	N/A	Down (65%)	Down (70%)
MEK3 (S189)	Same	Up (70%)	Same	Same	Same	Same
JNK (T183/Y185)	Same	Same	Down (100%)	Same	Up (50%)	Same
MSK1 (S376)	Same	Same	Same	Same	Same	Same
JUN (S73)	N/A	N/A	N/A	N/A	Same	Same
STAT3 (S727)	**Same**	**Up (60%)**	**Same**	**Down (60%)**	**Same**	**Up (50%)**
Rb1 (S780)	**Up (50%)**	**Up (60%)**	**Same**	**Down (55%)**	**Up (50%)**	**Up (50%)**
Rb1 (S807/S811)	**Up (80%)**	**Up (80%)**	**Down (55%)**	**Down (100%)**	**Up (50%)**	**Up (65%)**
p38α (T180/Y182)	N/A	N/A	Down (50%)	Same	Same	Same

The cells were treated for 15 min or 24 h with 50 ng/ml IGF-I, or were left untreated in SFM. The phosphorylation of proteins on designated residues was detected by "phospho-screen" assay (Kinexus, www.kinexus.ca). The IGF-I-induced upregulation (Up), downregulation (Down), or no change (Same) of protein phosphorylation was calculated relative to the status in SFM, based on densitometry values provided by Kinexus. Changes of less than 50% were considered insignificant. N/A, protein not activated at any time point. Several IGF-I signaling proteins exhibiting differential patterns of activation depending on the ERα status are highlighted. The tendencies presented in this table were observed in three independent assays.

MDA-MB-231 and MDA-MB-231/IGF-IR cells, while in MCF-7 and MCF-7/IGF-IR cells, IGF-I increased ERK1/2 phosphorylation only transiently (24).

To further address the role of ERα in IGF-I signaling, we re-expressed ERα in MDA-MB-231 cells. The re-introduction of ERα restored some of IGF-I functions, most notably, MDA-MB-231/ER cells were found to express higher levels of IRS-1 and were able to survive in IGF-I. These survival effects correlated with increased IRS-1 tyrosine phosphorylation, and sustained phosphorylation of Akt (S475) and GSK-3 α/β (S21/S9) kinases. However, the phosphorylation of IRS-2 remained unchanged (79).

In the next step, a comprehensive analysis of IGF-I signaling in ERα-positive and -negative cells was carried out using an antibody array proteomics screen (Table II and Fig.1). The screen has been developed to

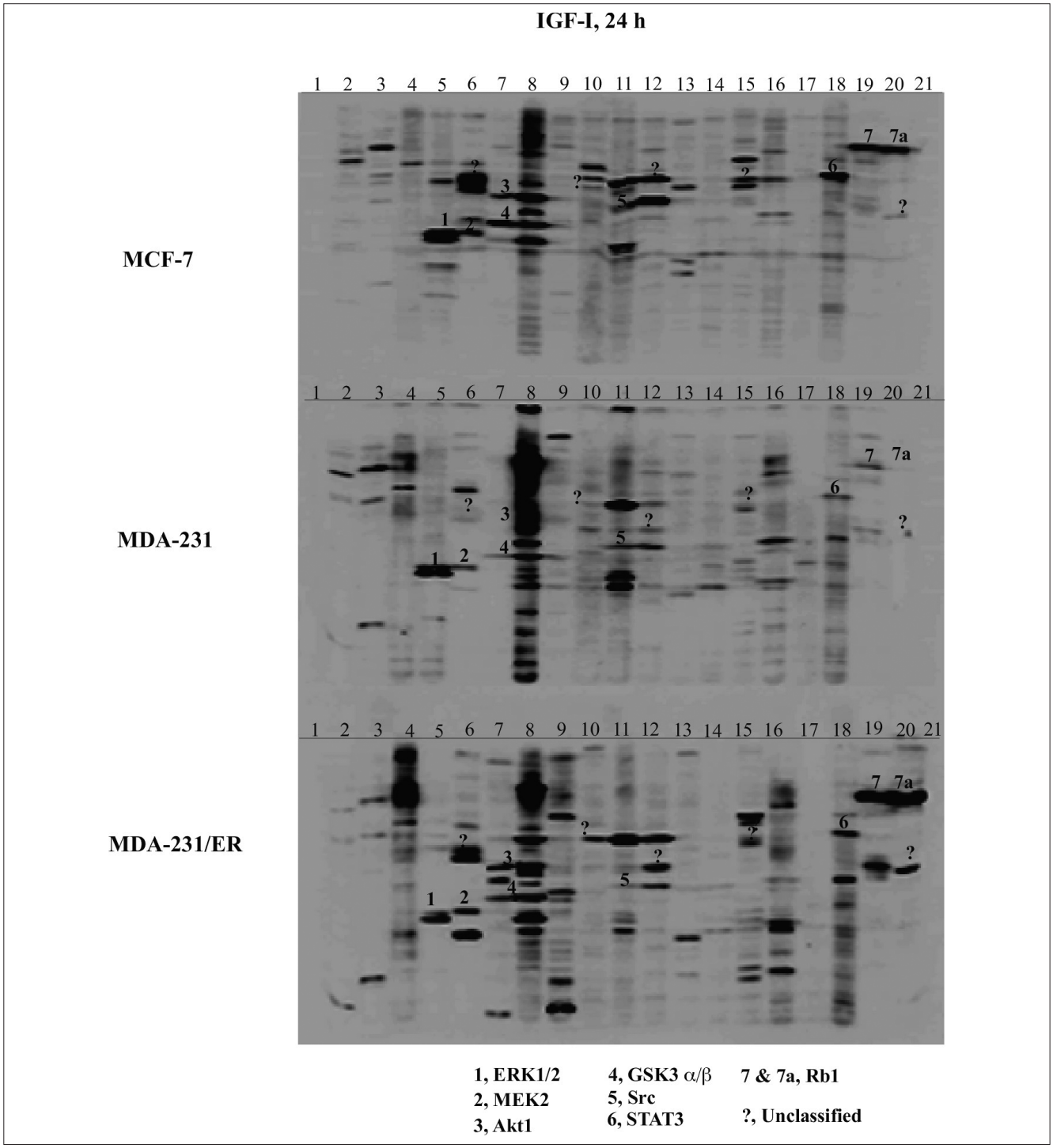

Fig. 1 - Representative pattern of activation of kinases and cell cycle regulators in ERα-positive and ERα-negative breast cancer cells stimulated with IGF-I. ERα-positive MCF-7 and MDA-MB-231/ER cells and ERα-negative MDA-MB-231 cells were synchronized in SFM, as described before (24). The cells were stimulated for 15 min and 24 h with 50 ng/ml IGF-I. Total cell lysates were prepared following Kinexus instructions and analyzed with Kinexus technology. The numbers above bands indicate several proteins differentially regulated by IGF-I in ERα-positive and ERα-negative cells (see Tab. II for details on phosphorylation sites and levels of activation): 1, ERK1/2; 2, MEK2; 3, Akt1; 4, GSK α/β; 6, STAT3; 7&7a Rb1; 8, Src; ?, unclassified proteins detected by Kinexus antibodies. The analysis was repeated three times, representative images obtained for 24 h stimulation assay are shown.

track the phosphorylation status of 33 known cellular kinases and other cellular regulators (www.kinexus.ca). Our goal was to identify IGF-I pathways whose activation was similar in MCF-7 and MDA-MB-231/ER cells and different in MDA-MB-231 cells. We observed that re-introduction of ERα was paralleled by a reversed activation pattern of several proteins, e.g., Akt-1, GSK-3 α/β, Rb1, STAT3 (Table II and Fig.1). Specifically, in MCF-7 and MDA-MB-231/ER cells IGF-I induced the phosphorylation of Akt1 (S473), GSK-3α/β (S21/S9), Rb1 (S807/811), and STAT3 (S727). In contrast, IGF-I treatment did not tyrosine-phosphorylate these proteins in MDA-MB-231 cells. It is worth noting that in the presence of ERα, IGF-I-dependent modulation of the above kinases and cell cycle regulators was consistent with the mitogenic pattern, while in the absence of ERα, it was consistent with growth inhibition. Thus, we tentatively classified Rb1 and STAT3 as signaling endpoints differentially regulated by IGF-I depending on the ERα status. Interestingly, in breast cancer, the expression of ERα positively correlates with Rb1 (101), while the association between ERα and STAT 3 has not been established (102). The mechanism by which ERα promotes IGF-I signaling to Rb1 could include increased IRS-1 expression, enhanced downstream signaling to Akt/GSK-3β, and accumulation of Cyclin D1 in ERα-positive cells. Indeed, E2-dependent stabilization of Cyclin D1 expression has been reported by Dufourny et al. (103). The intriguing possibility that IGF-I differentially regulates STAT3-mediated transcription in ERα-positive and ERα-negative breast cancer cells is currently under investigation in our laboratories.

Conclusions and Perspectives

The research of the past decade suggests the involvement of IGF-IR in breast cancer development. In this respect, tumorigenic activity of IGF-IR appears to be linked to hyperactivation of its anti-apoptotic and mitogenic signaling pathways due to overexpression of IGF-IR and/or IRS-1, induction of autocrine and paracrine IGFs, and possibly increased concentrations of endocrine ligands. The results obtained in breast cancer cell models suggest that mitogenic and anti-apoptotic effects of IGF-IR are characteristic for ERα-positive cells, while in the absence of ERα, IGF-I can produce non-mitogenic effects, such as migration. The elucidation of how the ERα status may dictate IGF-I responses will be critical in the development of anti-IGF-IR strategies for breast cancer.

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Inhibition of DNA Methylation Sensitizes Glioblastoma for Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand–Mediated Destruction

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Abstract

Life expectancy of patients affected by glioblastoma multiforme is extremely low. The therapeutic use of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) has been proposed to treat this disease based on its ability to kill glioma cell lines *in vitro* and *in vivo*. Here, we show that, differently from glioma cell lines, glioblastoma multiforme tumors were resistant to TRAIL stimulation because they expressed low levels of caspase-8 and high levels of the death receptor inhibitor PED/PEA-15. Inhibition of methyltransferases by decitabine resulted in considerable up-regulation of TRAIL receptor-1 and caspase-8, down-regulation of PED/PEA-15, inhibition of cell growth, and sensitization of primary glioblastoma cells to TRAIL-induced apoptosis. Exogenous caspase-8 expression was the main event able to restore TRAIL sensitivity in primary glioblastoma cells. The antitumor activity of decitabine and TRAIL was confirmed *in vivo* in a mouse model of glioblastoma multiforme. Evaluation of tumor size, apoptosis, and caspase activation in nude mouse glioblastoma multiforme xenografts showed dramatic synergy of decitabine and TRAIL in the treatment of glioblastoma, whereas the single agents were scarcely effective in terms of reduction of tumor mass, apoptosis induction, and caspase activation. Thus, the combination of TRAIL and demethylating agents may provide a key tool to overcome glioblastoma resistance to therapeutic treatments. (Cancer Res 2005; 65(24): 11469-77)

Introduction

Malignant gliomas are brain tumors arising from cells of the astrocytic lineage. Glioblastoma multiforme is the most aggressive malignant glioma (grade 4 astrocytoma; ref. 1) characterized by a median survival of 10 to 12 months. Extensive surgical resection is not curative due to the highly invasive capacity of glioblastoma multiforme cells into normal brain parenchyma. Moreover, glioblastoma multiforme is largely resistant to current treatments based on cytotoxic approaches targeting replicating DNA, such as chemotherapy or radiotherapy. Consequently, only a small minority of glioblastoma multiforme patients achieves long-term survival (2–4).

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Impaired apoptosis contributes to tumor development and resistance to therapy. Mammalian cell apoptosis results from the activation of two major pathways. The intrinsic pathway is generated by multiple signals, including radiation and chemotherapy. This apoptotic route involves the mitochondria-dependent activation of the initiator caspase-9, which in turn activates downstream executive caspases, such as caspase-3. The antiapoptotic members of the Bcl-2 family promote tumor formation and resistance to therapy by preventing the release of apoptogenic factors from mitochondria (5).

The extrinsic death receptor pathway is triggered by death ligands belonging to the tumor necrosis factor (TNF) family, such as CD95 ligand and TNF-related apoptosis-inducing ligand (TRAIL), through the formation of the death-inducing signaling complex (DISC). This complex is composed of aggregated death receptors, the adaptor molecule FADD, and the initiator caspase-8. After DISC formation, the zimogen form of caspase-8 is proteolytically cleaved and activated to initiate the apoptotic signaling (6, 7). Death receptor activation can be blocked by c-FLIP and PED/PEA-15, two inhibitory proteins that compete with caspase-8 for FADD binding and neutralize the extrinsic apoptotic pathway (8, 9).

Death receptor triggering can promote apoptosis independently from the mitochondrial pathway. Therefore, death receptor ligands may kill tumor cells resistant to chemotherapy and radiotherapy. Several defects within the apoptotic machinery have been identified in tumors of different origin. One of the mechanisms responsible for apoptosis resistance in cancer results from silencing of tumor suppressor or proapoptotic genes, occurring by hypermethylation of the CpG-rich sites located in the promoter region of the gene. Methyltransferases may contribute to the development of glioblastomas through the transcriptional inhibition of the carboxyl-terminal modulator protein, which binds Akt and reduces its protein kinase activity (10). The tumorigenic role of methyltransferases in brain tumors is further supported by the repression of caspase-8 expression observed in neuroblastomas and medulloblastomas, where the use of the methyltransferase inhibitor decitabine (5-aza-2-deoxycytidine) results in caspase-8 up-regulation and restoration of apoptosis sensitivity (11–16).

Due to its ability to induce apoptosis preferentially in cancer cells, the apoptotic pathway activated by TRAIL is a very attractive candidate for cancer treatment, currently exploited in several phase I trials through the use of recombinant TRAIL or agonistic anti–TRAIL receptor antibodies (17, 18).

The weak cytotoxic effect of chemotherapeutic drugs on glioblastoma multiforme cells encouraged several investigators to examine the sensitivity of glioblastoma cells to recombinant TRAIL.

Based on its ability to kill some glioblastoma cell lines both *in vitro* and *in vivo*, TRAIL has been proposed for glioblastoma treatment (19–23). However, glioblastoma cell lines exhibited variable sensitivity to TRAIL. In some cases, the combined administration of other compounds has been proposed for increasing TRAIL-induced apoptosis, such as chemotherapeutic drugs or cell-permeable peptides mimicking the mitochondrial release of the proapoptotic protein Smac/Diablo (24–26).

Little is known about primary glioblastoma cells and their response to TRAIL stimulation. Although the extreme variability of glioblastoma cell lines in terms of TRAIL sensitivity might reflect the heterogeneity of the tumors from which the cells have been derived, it cannot be excluded that such variability results from adaptation to *in vitro* growth. Direct analysis of tumors and primary cells is required to obtain more reliable data concerning the glioblastoma multiforme response to TRAIL. In this study, we investigated TRAIL signaling pathway in human glioblastomas. We found that primary glioblastoma cells were completely refractory to TRAIL stimulation. However, treatment with DNA demethylating agents was able to restore the sensitivity to TRAIL-induced apoptosis *in vitro* and *in vivo* through the reconstitution of the early signaling pathway.

Materials and Methods

Tumor cell isolation and characterization. Primary cultures of glioblastoma were established from specimens obtained from consenting patients undergoing surgery at the Department of Neurosurgery, Catholic University, Rome, Italy (Table 1). The institutional review board at the Catholic University approved this study. Tissues were mechanically disrupted in the presence of HBSS. Cell suspension was recovered, passed through 100-μm nylon cell strainers, and subjected to Ficoll gradient centrifugation. Cells were then cultured in DMEM/F-12 complete medium supplemented with 10% fetal bovine serum (FBS). Phenotypic characterization of isolated primary cells in comparison with a panel of glioblastoma cell lines was done by flow cytometry and real-time PCR. Cells isolated from all tumor samples homogeneously expressed the neural progenitor cell marker nestin and the glial fibrillary acidic protein (GFAP), whereas these proteins were differently expressed in the four cell lines. In agreement with literature data, both primary cultures and cell lines constantly expressed the neuron-specific enolase (27). Whereas the fibroblast antigen Thy1 was present in three of four glioblastoma cell lines, the absence of contaminant endothelial cells and fibroblasts in primary glioblastoma cells was confirmed by negativity for von Willebrand factor and Thy1, respectively (data not shown). Thus, primary cultures derived from tumor samples were virtually pure glioblastoma cultures.

The human glioblastoma cell lines T98G, U87MG, U251, and TB10 (28) were grown in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated FBS (Life Technologies, Inc., Grand Island, NY), 100 units/mL penicillin, 100 units/mL streptomycin, and 2 mmol/L L-glutamine (Life Technologies, Inc., Rockville, MD) and maintained in 5% CO_2 at 37°C.

Flow cytometric analysis. One hundred thousand cells were used for flow cytometric analysis. Cells were washed with cold PBS and incubated with control or specific antibodies. Mouse anti-Thy1 antibodies (PharMingen, Inc., San Diego, CA), goat anti–TRAIL receptor antibodies (R&D Systems, Minneapolis, MN), phycoerythrin-conjugated anti-goat secondary antibodies (Chemicon, Temecula, CA), and FITC-conjugated anti-mouse antibodies (Molecular Probes, Eugene, OR) were used. Labeled cells were washed twice with PBS and fluorescence intensity was evaluated by FACScan (Becton Dickinson, San Jose, CA).

Detection of apoptosis and caspase activation. Decitabine (Sigma, St. Louis, MO) was dissolved in DMSO 100 mmol/L, and 1-μL aliquots were stored at −20°C. Single aliquots were thawed immediately before use and diluted in complete medium. Cells were grown in the presence of 0.1 to 1 μmol/L decitabine for 6 days to be used for DNA demethylation experiments. Decitabine-containing medium was replaced daily. Cells were treated with leucine zipper TRAIL (LZ-TRAIL; kindly provided by Dr. Henning Walczak, Heidelberg, Germany) in complete medium for apoptosis induction. Cell viability was then analyzed by Cell Titer 96 assay (Promega, Madison, WI) and caspase activation was measured by Apo1 Caspase-3/7 Assay kit (Promega). Colorimetric or fluorimetric assays were analyzed by Victor 2 plate reader (Wallac, Turku, Finland).

Western blotting and real-time PCR analyses. For immunoblotting studies, mouse monoclonal anti-FADD/MORT1 antibody was purchased from Becton Dickinson Transduction (Los Angeles, CA) and mouse monoclonal anti-caspase-8 (clone 5F7) was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-PED/PEA-15 antibody was a kind gift of G. Condorelli (Naples, Italy). Mouse monoclonal anti-β-tubulin antibody was purchased from Sigma. Bands were detected with Super Signal West Pico chemiluminescent substrates (Pierce, Rockford, IL) and quantified using Scion Image software (Scion Corp., Frederick, MA). For real-time PCR, total RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA (1 μg) was reverse transcribed into cDNA by using SuperScript II RT with oligo(dT) as primers (Invitrogen, Grand Island, NY) according to the manufacturer's protocol.

Table 1. Clinical features and immunohistochemical pattern of glioblastoma tumors

Case code	Age/sex	Tumor location	Immunohistochemistry			
			GFAP	Ki-67 (%)	p53	Epidermal growth factor receptor
GBM2	60/F	Temporal	+	10	Wild-type	+
GBM3	55/F	Parietal	+	30	Mutant	+
GBM5	62/F	Frontal	+	10	Wild-type	+
GBM6	40/M	Parietal	+	15	Mutant	−
GBM7	35/M	Temporal	+	15	Mutant	−
GBM8	55/F	Frontal	+	10	Mutant	+
GBM9	66/F	Temporal	+	15	Mutant	+
GBM10	67/M	Frontal	+	20	Mutant	+
GBM11	73/M	Frontal	+	18	Wild-type	+

NOTE: Immunohistochemical pattern of glioblastoma tumors. Expression analysis of specific proteins was done by immunohistochemistry.

Real-time PCR was done with ABI Prism 7900HT Sequence Detection System and all reagents were from Applied Biosystems (Foster City, CA) following the manufacturer's instructions. Assays-on-Demand for TRAIL receptor-1 (TRAIL-R1), PED/PEA-15, and caspase-8 were used.

Retroviral gene transfer. Caspase-8 coding sequence and antisense PED/PEA-15 sequence were cloned into PINCO retroviral vector. TRAIL-R1 cDNA was cloned into a green fluorescent protein (GFP)–defective PINCO vector. Retroviral particle generation and tumor cell infection was done as described (29). The evaluation of infection efficiency was done by flow cytometry based on the expression of the GFP reporter protein or TRAIL-R1 immunostaining preceded flow cytometric analysis in the case of GFP-defective vector. The percentage of infected cells was >95%. Caspase-8 overexpression and PED/PEA-15 down-modulation were evaluated by Western blotting analysis.

Animal studies and immunohistochemistry of tumor tissues. One million U87MG glioblastoma cells were injected s.c. into one flank of 6-week-old athymic *nu/nu* mice (Charles River Laboratory, Wilmington, MA). The use and care of experimental animals was approved by the ethical committee of the Catholic University School of Medicine (Rome, Italy). Mice were kept under pathogen-free conditions and observed daily for the visual appearance of tumors at injection sites. Tumor diameter was measured using calipers and calculated as the mean value between the shortest and the longest diameters. When tumors reached ~6 mm in mean diameter (~3-4 weeks postinjection), treatment of mice with decitabine was started. Decitabine (3.75 mg/kg) was administered i.p. twice daily for 6 days. Two injections of 2 μg LZ-TRAIL at the tumor site were done after 4 and 6 days of decitabine treatment. Mice were maintained up to 11 days without any further treatment, except for measurement of tumor masses. Control animals were injected with equal volumes of saline either i.p. or at the tumor site.

For *in situ* apoptosis detection in tumor xenografts, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) reaction was done using *In situ* Cell Death Detection AP kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. Apoptotic nuclei appeared as blue dots. Counterstaining of cytoplasm was done using eosin.

Immunofluorescence staining was done on 6-μm-thick paraffin-embedded tumor xenograft sections. Tissue samples were deparaffinized and hydrated. For antigen unmasking, sections were heated in 10 mmol/L sodium citrate buffer (pH 6.0) and washed in distilled H_2O for 5 minutes. Slides were then incubated for 5 minutes in 1% H_2O_2, washed, and exposed to 1% bovine serum albumin (BSA)–containing TBS for 10 minutes to reduce unspecific staining. Excess BSA was removed and samples were incubated with active caspase-8-specific rabbit polyclonal antibody or isotype-matched control antibody (Cell Signaling Technology, Beverly, MA) overnight at 4°C. After two washes in TBS, sections were exposed to rhodamine-conjugated goat anti-rabbit immunoglobulins (Molecular Probes). Nuclei were labeled with Hoechst 33342 (Molecular Probes).

For immunohistochemical staining, 5-μm-thick paraffin-embedded sections were incubated with TBS/BSA for 10 minutes to reduce unspecific staining. Tissue sections were then exposed to goat polyclonal antibody against caspase-8 (N19, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or isotype-matched control antibody for 1 hour. After two washes in TBS, sections were exposed to anti-goat biotinylated antibody, washed again, and incubated with streptavidin-conjugated peroxidase (Vectastain, Universal Quick kit, Vector Laboratories, Inc., Burlingame, CA). Peroxidase activity was revealed with 3-amino-9-ethylcarbazole as a substrate. Counterstaining of tissue sections was done using aqueous hematoxylin.

Results

Low levels of caspase-8 and high levels of PED/PEA-15 correlated with resistance of primary glioblastoma cells to tumor necrosis factor–related apoptosis-inducing ligand–induced death. To determine whether the cancer cells forming human glioblastoma multiforme can be killed by TRAIL, isolated primary glioblastoma cells from surgical specimens were compared with four glioblastoma cell lines for sensitivity to TRAIL-induced apoptosis. As expected, glioblastoma cell lines displayed variable responses to TRAIL stimulation. T98G, U87MG, and U251 cells were partially sensitive to TRAIL-induced apoptosis, whereas TB10 cells were completely resistant (Fig. 1*A*). Surprisingly, primary cells from all the nine patients analyzed were refractory to TRAIL stimulation and did not undergo apoptosis even if exposed to high doses of recombinant TRAIL (Fig. 1*B*).

To explore the mechanisms responsible for TRAIL resistance, we investigated whether an altered expression of key elements of the proximal TRAIL pathway could be involved in impaired transmission of the apoptotic signal. We first analyzed by flow cytometry the expression levels of TRAIL receptors both in cell lines and in primary glioblastoma cells. Whereas TRAIL-R1 was weakly expressed, TRAIL receptor-2 (TRAIL-R2) was present at higher levels in all the cells analyzed (Fig. 1*C*). The expression levels of TRAIL-R1 and TRAIL-R2 were similar in both TRAIL-sensitive and TRAIL-resistant cells, indicating that the levels of TRAIL receptors may be unrelated to the different TRAIL susceptibility. We therefore investigated the expression of other TRAIL-related apoptotic and antiapoptotic proteins. FADD was consistently expressed in all the cell lines analyzed, ruling out its possible involvement in the different response to TRAIL stimulation (Fig. 1*D*). In contrast, caspase-8 immunoblot analysis showed that caspase-8 was expressed at very low levels in all the primary cells analyzed and in the TRAIL-resistant cell line TB10, whereas the partially sensitive cell lines expressed higher levels of caspase-8, suggesting that TRAIL resistance of glioblastoma cells resulted from insufficient caspase-8 activation (Fig. 1*D*). Moreover, the levels of the caspase-8 inhibitory protein PED/PEA-15 were higher in resistant than in sensitive cells, whereas the antiapoptotic protein c-FLIP was expressed at very low levels in all the cells analyzed (Fig. 1*D*; data not shown). Thus, the sensitivity to TRAIL-induced apoptosis in glioblastoma cells seems to be related to the ratio between the levels of caspase-8 and PED/PEA-15, which was considerably higher in sensitive cells (Fig. 1*D*).

To exclude the possibility that the low caspase-8 expression detected in glioblastoma primary cultures was dependent on *ex vivo* manipulation of glioblastoma cells, we investigated the expression of caspase-8 directly in tumors obtained at surgery from patients. In agreement with *in vitro* results, immunohistochemical analysis has clearly proven that glioblastomas display very low levels of caspase-8 and high expression of PED/PEA-15 (Fig. 1*E*), confirming the data obtained in cells from disaggregated tumors. Thus, the death receptor pathway is not functional in glioblastomas.

Decitabine treatment of primary glioblastoma cells results in sensitization to tumor necrosis factor–related apoptosis-inducing ligand–induced death. The expression of caspase-8 can be impaired by the activity of methyltransferases (11–16). Therefore, we investigated whether treatment with the methylation-defective cytidine analogue decitabine could restore the sensitivity to TRAIL-induced death in primary glioblastoma cells. Exposure to decitabine resulted in a considerable dose-dependent growth reduction of glioblastoma cells (Fig. 2*A*). Cell death analysis revealed that, although in the presence of 0.1 μmol/L decitabine cell viability was preserved, a significant number of dead cells were detectable at higher doses (data not shown). To reduce the unspecific toxicity, 0.1 μmol/L decitabine was used in all subsequent *in vitro* studies. As observed previously in other tumors (30, 31), decitabine-mediated growth inhibition correlated with

higher levels of p21 (Fig. 2*B*), a methyltransferase-regulated cell cycle inhibitor that binds to cyclin/cyclin-dependent kinase complexes and blocks cell proliferation (32).

Following exposure to decitabine, all partially sensitive cell lines displayed increased induction of cell death on TRAIL receptor stimulation. More importantly, those glioblastoma cells that were completely resistant acquired substantial sensitivity to TRAIL killing (Fig. 2*C*; data not shown). The restored TRAIL-induced apoptosis in primary glioblastoma cells treated with decitabine was associated with a considerable increase in caspase activation, which was barely detectable in the absence of methyltransferase inhibition (Fig. 2*D*). Thus, treatment with decitabine primes primary glioblastoma cells for TRAIL-mediated apoptosis.

Decitabine treatment of primary glioblastoma cells results in up-regulation of tumor necrosis factor–related apoptosis-inducing ligand receptor 1 and caspase-8 and down-modulation of PED/PEA-15. We then explored the mechanism of decitabine-induced TRAIL sensitization of primary glioblastoma cells. Reportedly, treatment of glioblastoma cell lines with chemotherapeutic agents resulted in increased TRAIL-R2 expression (33). Therefore, we first evaluated the possible modulation of those receptors for TRAIL able to transduce apoptotic signals.

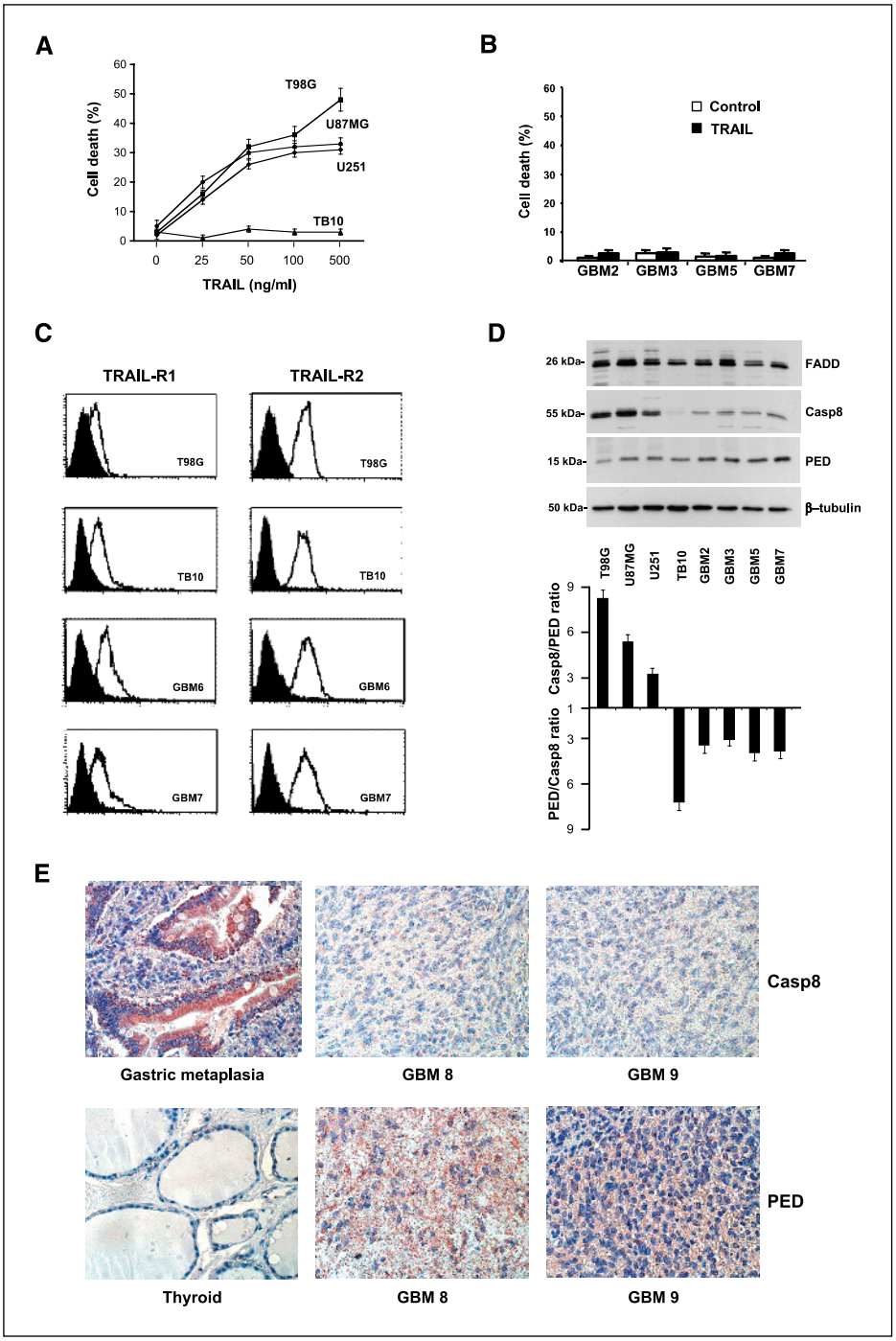

Figure 1. Sensitivity of glioblastoma multiforme to TRAIL-mediated cytotoxicity. Evaluation of cell death in glioblastoma cell lines (*A*) or primary glioblastoma cells (indicated as GBM followed by a number) from four different patients (*B*) exposed to different doses (*A*) or 500 ng/mL LZ-TRAIL (*B*). *Points,* mean of three independent experiments; *bars,* SD (*A*). *Columns,* mean of three independent experiments; *bars,* SD (*B*). *C,* flow cytometric analysis of TRAIL-R1 and TRAIL-R2 expression in the indicated cell lines and primary glioblastoma cells. *White histograms,* specific TRAIL receptor staining; *black histograms,* fluorescence controls. *D,* immunoblot analysis of caspase-8 (*Casp8*), FADD, and PED/PEA-15 (*PED*) expression in the glioblastoma cells analyzed in (*A* and *B*). β-Tubulin was used as loading control (*top*). *Columns,* mean absorbance of immunoblot band ratios obtained in four different experiments; *bars,* SD (*bottom*). *E,* immunohistochemical analysis of caspase-8 and PED/PEA-15 in two representative of four glioblastoma multiforme from different patients. Gastric metaplasia and nonneoplastic thyroid specimens were used as positive and negative controls, respectively. Representative of similar independent experiments with cells from nine patients (*B-E*).

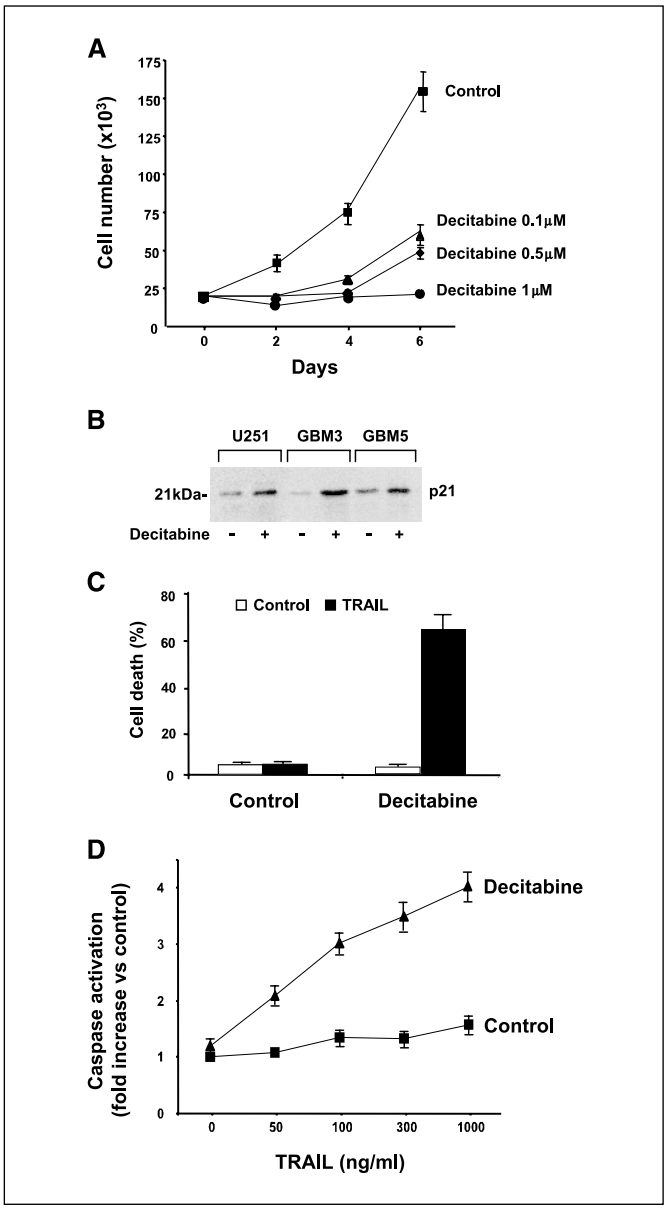

Figure 2. Decitabine-mediated sensitization to TRAIL in glioblastoma cells. *A,* primary glioblastoma cell growth in the presence of different doses of decitabine. *B,* immunoblot analysis of p21 expression in glioblastoma cells untreated (-) or treated (+) with decitabine. *C,* percentage of cell death in primary glioblastoma multiforme cells treated with 500 ng/mL LZ-TRAIL. *D,* LZ-TRAIL-induced caspase activation in control or decitabine-treated primary glioblastoma cells as determined by fluorescent caspase substrate cleavage. Caspase activation and cell death were measured 48 hours after LZ-TRAIL stimulation. *Points,* mean of five independent experiments with primary cells from seven different patients; *bars,* SD.

Control and decitabine-treated cell lines and primary glioblastoma cells were compared by flow cytometry for TRAIL-R1 and TRAIL-R2 expression. Although TRAIL-R1 was weakly expressed in untreated glioblastoma cells, its expression was considerably increased in decitabine-treated cells, suggesting that DNA methylation events might regulate TRAIL-R1 transcription in glioblastoma. In contrast, TRAIL-R2 was expressed at high levels in control cells and did not undergo any significant variation after decitabine treatment (Fig. 3*A*). We next investigated whether inhibition of DNA methylation could result in modulation of the intracellular components of the TRAIL pathway altered in glioblastoma. Primary glioblastoma multiforme cells were treated with decitabine and analyzed by immunoblot analysis for expression of caspase-8 and PED/PEA-15. Decitabine treatment of both cell lines and primary cells resulted in marked caspase-8 up-regulation and down-regulation of PED/PEA-15, whereas FADD expression was not significantly modified (Fig. 3*B*). The correlation between caspase-8 up-regulation, PED/PEA-15 down-regulation, and increased sensitivity to TRAIL suggested that decitabine treatment allowed efficient DISC formation after TRAIL stimulation.

To evaluate whether variations in the protein levels observed after decitabine treatment were dependent on transcriptional events, we measured caspase-8, TRAIL-R1, and PED/PEA-15 mRNA by real-time PCR analysis. Control and decitabine-treated TRAIL-resistant TB10 cells were compared with TRAIL-sensitive T98G cells. Whereas decitabine-treated samples displayed increased TRAIL-R1 and caspase-8 mRNA levels, PED/PEA-15 levels in the control samples were not significantly different from those in the treated samples (Fig. 3*C*). In particular, caspase-8 mRNA levels increased by 5.6-fold. A similar pattern was observed in primary glioma cells from three patients, where decitabine treatment resulted in a significant increase ($\sim 4.8 \pm 0.5$–fold) in caspase-8 mRNA (data not shown). Thus, whereas TRAIL-R1 and caspase-8 expression is regulated at the transcriptional level by decitabine treatment, the decrease in PED/PEA-15 levels may not be directly dependent on transcriptional regulation, possibly resulting from altered protein stability (34).

Caspase-8 up-regulation is the major event responsible for decitabine-mediated tumor necrosis factor–related apoptosis-inducing ligand sensitization. To evaluate the relevance for TRAIL sensitization of single proteins modulated by decitabine, we reproduced these protein level modifications in primary glioblastoma cells using retroviral vectors. Overexpression of TRAIL-R1 was not able to sensitize primary glioblastoma cells to TRAIL (Fig. 4*A* and *B*), suggesting that decitabine-mediated up-regulation of this receptor is unable to promote TRAIL cytotoxicity in the absence of substantial levels of caspase-8. Similarly, antisense cDNA-mediated reduction of PED/PEA-15 at levels comparable with those obtained with decitabine treatment did not prime these cells for TRAIL killing (Fig. 4*C* and *D*). In contrast, exogenous expression of caspase-8 was sufficient to promote caspase activation and apoptosis in primary glioblastoma cells exposed to TRAIL (Fig. 4*D* and *E*), indicating that caspase-8 up-regulation is a major event in decitabine-induced TRAIL sensitization.

Combined treatment with decitabine and tumor necrosis factor–related apoptosis-inducing ligand results in dramatic reduction of tumor growth and induction of tumor cell apoptosis in human glioblastoma xenografts. To evaluate the *in vivo* effectiveness of decitabine-mediated TRAIL sensitization of glioblastoma multiforme cells, we compared the antitumor activity of decitabine, TRAIL, or the combination of both agents in U87MG s.c. xenograft mouse model system (35). When tumors reached ~ 6 mm in size, mice were treated with decitabine alone or in combination with LZ-TRAIL. Decitabine was administered i.p. for 6 days and LZ-TRAIL was injected locally at days 4 and 6 of decitabine treatment. As a control, PBS was injected i.p. and i.t. Tumors were measured daily up to day 11, when the mice were killed to allow microscopic analysis of the

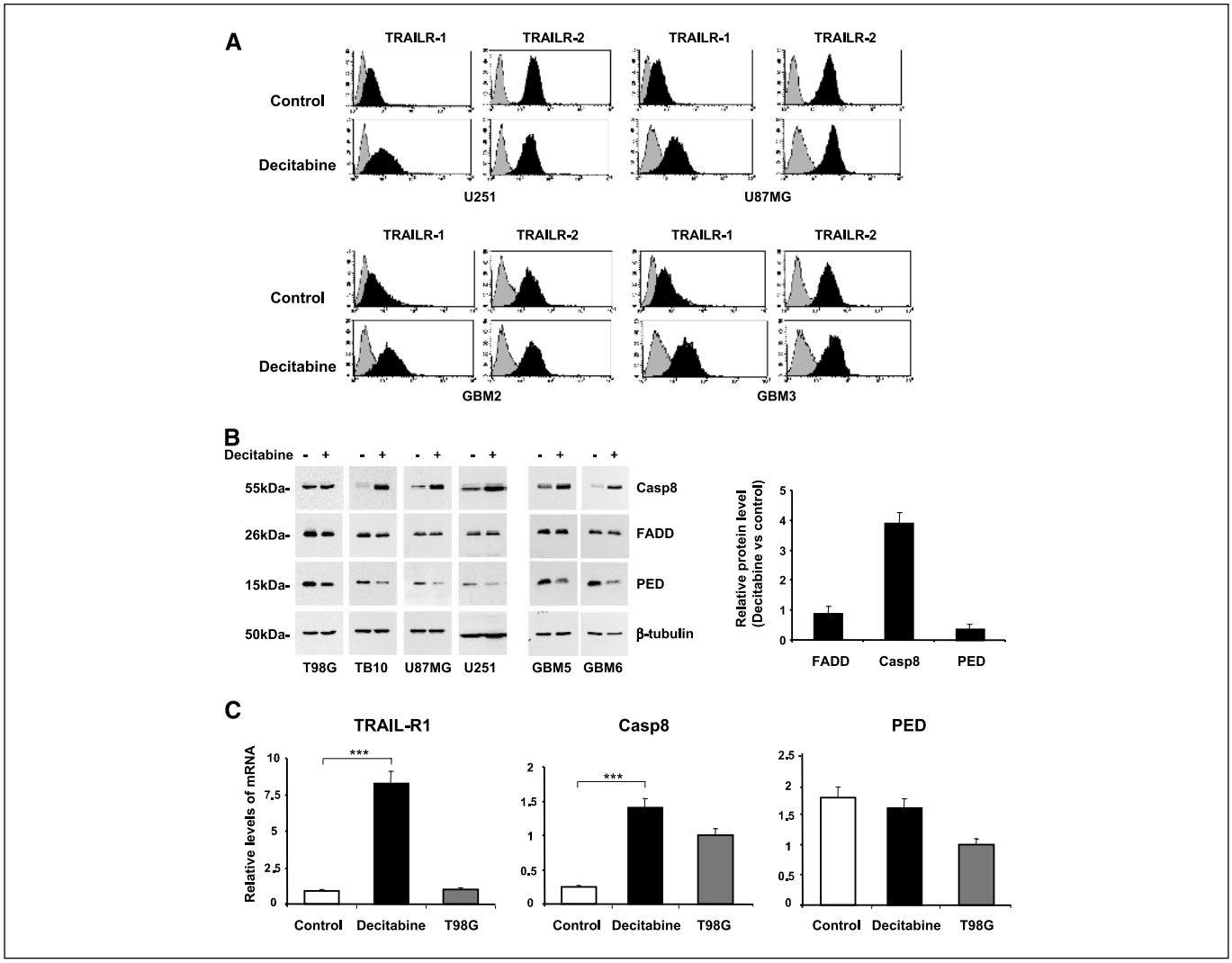

Figure 3. Up-regulation of TRAIL-R1 and caspase-8 and down-regulation of PED/PEA-15 by decitabine treatment. *A,* detection of TRAIL-R1 and TRAIL-R2 expression by flow cytometry in control or decitabine-treated primary glioblastoma cells. *Black histograms,* specific TRAIL receptor staining; *gray histograms,* fluorescence controls. *B,* immunoblot analysis of caspase-8, FADD, and PED/PEA-15 expression in glioblastoma cells untreated (-) or treated (+) with decitabine. β-Tubulin was used as loading control. *Columns,* mean of four experiments evaluating absorbance of immunoblot band ratios between treated and untreated primary cells from four different patients; *bars,* SD. Data were normalized for β-tubulin expression. *C,* real-time PCR analysis of TRAIL-R1, caspase-8, and PED/PEA-15 expression in TRAIL-resistant TB10 cells untreated (*Control*) or treated with decitabine. TRAIL-sensitive T98G glioblastoma cell line cDNA was used as positive control. ***, $P < 0.0001$.

tumor tissue. In agreement with *in vitro* experiments, decitabine treatment promoted a considerable up-regulation of caspase-8 in tumor xenografts (Fig. 5*A*). Macroscopic analysis showed that treatment with either decitabine or TRAIL alone resulted in modest reduction of final tumor size (Fig. 5*B*) possibly secondary to the transient inhibition of tumor cell proliferation rather than induction of apoptosis as suggested by the low number of apoptotic cells observed *in vivo* (Fig. 5*C*). In contrast, the combined administration of decitabine and TRAIL was able to induce a marked and consistent reduction of tumor size (Fig. 5*B*) and induction of apoptosis (Fig. 5*C*). Accordingly, whereas active caspase-8 was rarely detected in tumor xenografts treated with either single agent, the treatment with decitabine and TRAIL resulted in massive caspase-8 activation (Fig. 5*D*), confirming the effective triggering of the TRAIL death pathway. Moreover, H&E staining revealed that tumors of mice treated with decitabine and TRAIL were largely degenerated, showing massive postapoptotic and necrotic areas not observed in tumors treated with either single agent (data not shown). These results indicate that the combined administration of decitabine and TRAIL is able to induce a remarkable antitumor effect *in vivo* through the inhibition of cell growth and the induction of apoptosis in glioblastoma multiforme cells.

Discussion

The poor prognosis of malignant gliomas calls for intensive molecular and preclinical investigations to develop new and effective therapies. Current nonsurgical cancer treatments are essentially based on radiotherapy or chemotherapy, which exploit the intrinsic apoptotic pathway to destroy the tumor. Therefore, the majority of therapy-resistant cancers have an upstream or downstream defect involving the intrinsic apoptotic pathway. The possibility to exploit the extrinsic pathway in cancer

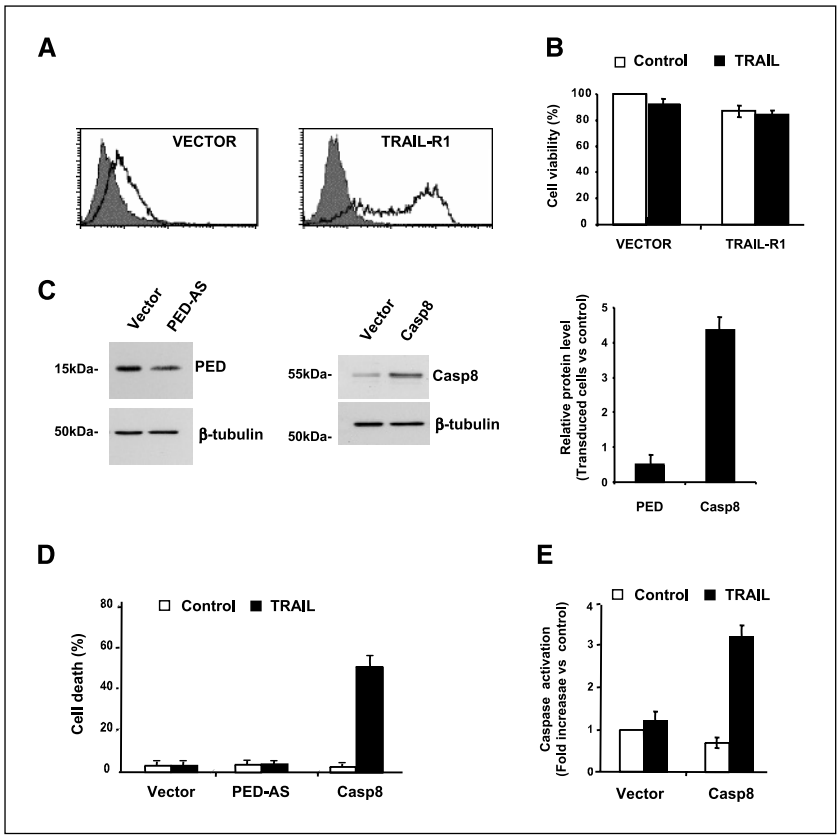

Figure 4. Effect of exogenous gene expression on TRAIL-sensitivity. *A,* flow cytometric analysis of TRAIL-R1 in primary glioblastoma cells transduced with empty vector or TRAIL-R1 cDNA. *B,* percentage of viability in cells transduced as in (*A*) and treated with LZ-TRAIL. *C,* PED/PEA-15 and caspase-8 expression in primary glioblastoma cells transduced with empty vector, PED/PEA-15 antisense (*PED-AS*), or caspase-8. Relative protein levels were quantified by densitometry. LZ-TRAIL induced cell death (*D*) and caspase activation (*E*) of primary glioblastoma cells transduced as indicated. Cell death and caspase activation were measured 48 hours after LZ-TRAIL stimulation. *Columns,* mean of three independent experiments with GBM6, GBM7, and GBM8 primary cells; *bars,* SD.

treatment has become feasible after the discovery that TRAIL may be administered to patients based on its ability to preferentially induce apoptosis in cancer while sparing normal cells (17, 18).

Several studies have shown that TRAIL is able to kill different glioma cell lines (20, 21, 23). Following the inability of chemotherapy and radiotherapy to improve patient prognosis, TRAIL has been proposed as an attractive candidate for glioblastoma treatment. However, some cell lines were reported to be resistant to TRAIL-induced apoptosis. Such resistance has been proposed to depend on high levels of antiapoptotic protein PED/PEA-15 expression (23).

In this study, we showed that primary glioblastoma multiforme cells are completely refractory to TRAIL-mediated apoptosis. However, treatment with decitabine was able to restore their responsiveness to TRAIL stimulation through caspase-8 and TRAIL-R1 up-regulation and down-regulation of PED/PEA-15. The synergistic activity of decitabine and TRAIL was confirmed *in vivo* using a mouse xenograft model, which showed massive apoptotic regression of treated tumors.

In agreement with other studies, we found a variable sensitivity to TRAIL in glioma cell lines (20, 21, 23). In contrast, all primary cells analyzed were invariably resistant to TRAIL-mediated apoptosis. It is likely that results achieved with the use of primary cells are more reliable than those obtained with cell lines as shown by the extremely low levels of caspase-8 and high PED/PEA-15 expression consistently observed by immunohistochemistry in tumors. The heterogeneity of glioblastoma cell lines was evident even during the phenotypical analysis of basic glioma markers. Whereas the four cell lines analyzed displayed significant variability in terms of antigen expression with GFAP, neuron-specific enolase, nestin, and the fibroblast antigen Thy1 being expressed at different levels, primary cells were rather homogeneous.

In agreement with the studies analyzing glioma cell lines (23), we observed that high levels of the antiapoptotic protein PED/PEA-15 correlated with increased resistance to TRAIL. The balance between the expression of caspase-8 and its inhibitor PED/PEA-15 seems extremely relevant for determining the susceptibility to TRAIL-mediated apoptosis. Inhibition of DNA methylation in glioblastoma multiforme cells resulted in a modification of this balance in favor of caspase-8, thus increasing apoptosis susceptibility. Although methyltransferase-mediated caspase-8 silencing occurs in other cancers, there is a lack of agreement concerning the identification of the promoter responsible for epigenetic regulation of caspase-8 expression. Therefore, the significance of direct caspase-8 promoter demethylation or transacting factors acting on this promoter in decitabine-induced caspase-8 up-regulation remains to be determined.

We found that DNA demethylation also resulted in increased TRAIL-R1 expression. Differently from caspase-8, epigenetic silencing of TRAIL-R1 has not been observed in other neurologic tumors. However, low expression of TRAIL receptors in some cancers seems to be involved in resistance to TRAIL. Decitabine-induced TRAIL-R1 expression could represent a possible sensitization strategy to treat these types of cancer. For instance, low levels of TRAIL-R1 expression seem to be associated with TRAIL resistance in non–small cell lung carcinoma cells. A combined treatment with decitabine and IFN-γ was reported to increase both TRAIL-R1 levels and apoptosis sensitivity of these cells,

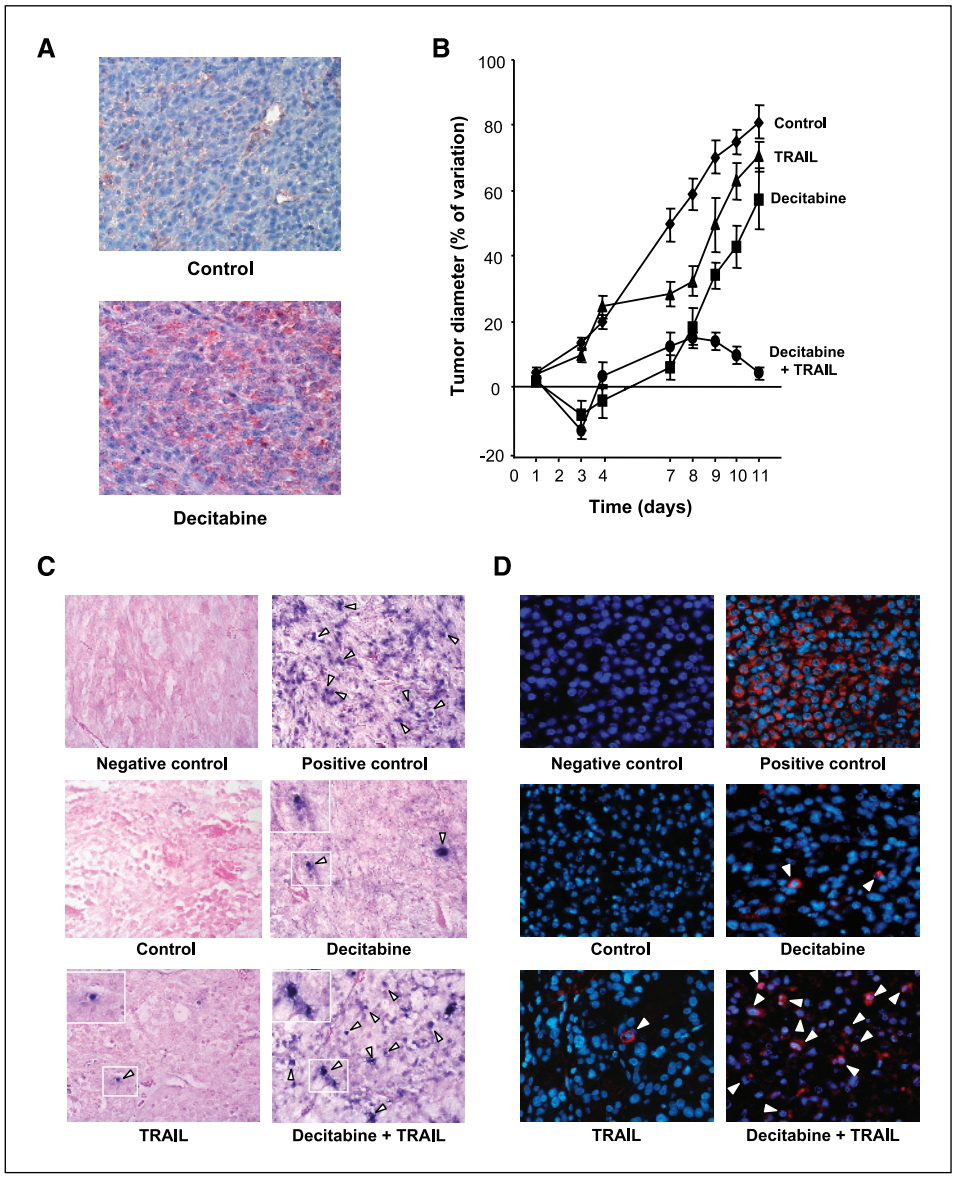

Figure 5. Effect of decitabine and TRAIL treatment on human glioblastoma xenografts. *A,* immunohistochemical analysis of caspase-8 expression done on U87MG xenografts derived from control or decitabine-treated mice. *B,* variation of tumor diameter at the indicated time points versus tumor diameter before decitabine treatment (day 0) in control mice or in mice treated with LZ-TRAIL, decitabine, or both compounds. *Points,* mean of two independent experiments, each experiment being composed by five mice per group (total of 40 animals); *bars,* SE. Tumors from mice treated with decitabine and LZ-TRAIL were significantly smaller than tumors from any other group (day 11; $P < 0.001$). *C, in situ* apoptosis detection in tumor xenografts by TUNEL reaction. Tumors were obtained at day 11 from mice untreated or treated with LZ-TRAIL, decitabine, or the combination of both compounds (*Decitabine + TRAIL*). *Arrowheads,* representative apoptotic cells stained in *dark blue. D,* immunofluorescence analysis of active caspase-8 of tumors obtained as in (*C*). Isotype-matched or active caspase-8-labeled tonsil sections were used as negative and positive controls, respectively.

indicating that epigenetic control of TRAIL-R1 transcription might occur in some other cancers (36).

Decitabine has been used in humans for the treatment of myelodysplastic syndromes, leukemia, and solid tumors. Phase I and II trials showed that decitabine is well tolerated and moderately effective in some types of cancer (37–39). To date, no data are available for clinical toxicity of TRAIL and agonist TRAIL receptor antibodies, which are currently undergoing phase I and II studies. However, experimental data are very promising in terms of antitumor activity and lack of toxicity. Here, we provide preclinical evidence for the efficacy of decitabine and TRAIL combination. Intense effort is required to assess the possible clinical use of decitabine and TRAIL combination for the treatment of glioblasvtoma given the high malignancy and low life expectancy of these patients.

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