Effects of Transferrin Receptor Blockade on Cancer Cell Proliferation and Hypoxia-Inducible Factor Function and Their Differential Regulation by Ascorbate

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Abstract
Cellular iron is needed for cell survival and hydroxylation of hypoxia-inducible factor-1α (HIF-1α) by prolyl hydroxylases (PHD). One mechanism of iron uptake is mediated by the cell surface transferrin receptor (TfR). Because iron is required for cell growth and suppression of HIF-1α levels, we tested the effects of the two anti-TfR monoclonal antibodies (mAb) E2.3 and A27.15 on growth of breast cancer cells and induction of HIF-1α and hypoxia-regulated genes. Treatment with both mAbs together synergistically inhibited cell proliferation in a dose-responsive manner by up to 80% following 8 days of exposure, up-regulated HIF-1α and HIF transcription targets, down-regulated TfR expression, and down-regulated cellular labile iron pool by 60%. Because combined treatment with anti-TfR mAbs resulted in the up-regulation of the hypoxia pathway, which may increase tumor angiogenesis, we analyzed the effects of ascorbate on cell viability and HIF-1α levels in cells treated with both anti-TfR mAbs together, as ascorbate has been shown to be required by PHD enzymes for full catalytic activity. Ascorbate at physiologic concentrations (25 μmol/L) suppressed HIF-1α protein levels and HIF transcriptional targets in anti-TfR mAb-treated cells but did not suppress the antiproliferative effect of the mAbs. These results indicate that the addition of ascorbate increased the activity of the PHD enzymes in down-regulating HIF but not the proliferation of iron-starved anti-TfR mAb-treated cells. The use of anti-TfR mAbs and ascorbate in inhibiting both cell proliferation and HIF-1α and angiogenesis under normoxic conditions may be of therapeutic use. (Cancer Res 2006; 66(5): 2749-56)

Introduction
Hypoxia-inducible factor (HIF) is an α,β heterodimeric transcription factor, which directs a broad range of responses in hypoxic cells (1). HIF-1α is a constitutive nuclear localized subunit, which binds to available HIF-α (2). In the presence of oxygen, two prolyl sites within a central degradation domain of HIF-α are hydroxylated by a set of closely related Fe(II) and 2-oxoglutarate-dependent oxygenases [prolyl hydroxylases (PHD) 1-3], which leads to HIF-α degradation via the pVHL E3 ubiquitin ligase complex and the 26S proteosome (3). A second hydroxylation-dependent control of HIF on an asparaginyl residue in the COOH-terminal activation domain by another Fe(II) and 2-oxoglutarate-dependent oxygenase, factor-inhibiting hypoxia, inhibits HIF-α transcriptional activity by preventing interaction with the p300/CBP coactivator (4). These enzymes are also known to be dependent on iron and ascorbate for full catalytic activity (3). Limiting oxygen levels or the availability of iron with iron chelators allows HIF-α to escape proteolysis and become transcriptionally active, which leads to up-regulation of genes involved in angiogenesis, glucose metabolism, and pH regulation (5). The HIF transcription cascade has been shown to contribute to tumor progression and metastasis and plays an important part in the malignant phenotype, contributing to increased angiogenesis, enhanced glycolysis, and other properties that promote growth (1).

In addition to iron involvement in hydroxylation of HIF-α, iron plays critical roles in electron transport and cellular respiration, cell proliferation and differentiation, and regulation of gene expression and DNA synthesis (6–8). Growing cells require iron to maintain activity of ribonucleotide reductase, which synthesizes deoxynucleotides for DNA synthesis. Iron in the serum is bound to the iron transport protein transferrin, and uptake of iron by cells is mediated by a cell surface transferrin receptor (TfR; ref. 9). Although TfR expression is relatively limited in normal tissues, many tumor cells display high levels on their surface (10–12). Therefore, TfR could be a relevant target for antibody-based therapies against tumors. Several authors have reported therapeutic approaches based on this idea using anti-TfR antibodies to kill malignant cells (13–15). It has been shown that combinations of monoclonal antibodies (mAb) against TfR on human tumor cells have antiproliferative effects in vitro and in vivo (16–18). However, targeting TfR, by reducing iron uptake, could reduce PHD activity, as PHDs are dependent on iron for full catalytic activity in suppressing HIF-1α levels. This raised whether the labile iron pool (LIP) regulated by TfR affects the HIF system in cells treated with anti-TfR mAbs. This could contribute to a self-limitation for therapeutic purposes. Because ascorbate is required by PHDs for catalytic activity and can enhance their activity, we wanted to assess if ascorbate could restore full PHD activity, even with low iron pools, and whether this would counteract the antitumor effects. Here, we report the effects of anti-TfR mAbs A27.15 and E2.3 on growth inhibition of human epithelial breast cancer cell lines and induction of HIF-1α and HIF transcriptional response and the effects of ascorbate on cells treated with anti-TfR mAbs.

Materials and Methods
Cell culture. Breast carcinoma MDA-468, MDA-231, and MCF-7 cell lines were obtained from the Cancer Research UK Cell Service and maintained in DMEM supplemented with 10% fetal bovine serum, 2 μmol/L L-glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin sulfate. L-Ascorbic acid sodium salt was obtained from Sigma (Poole, United Kingdom). Anti-TfR mAbs A27.15 and E2.3 were provided as a kind gift by The Salk Institute.
and isotype IgG1 antibody (Abcam, Cambridge, United Kingdom) was used as control.

Cell viability by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium, inner salt. Cells were seeded at 2.5 × 10^4 per well (100 μL) in 96-well plates 24 hours before experimental treatments. Cells were treated with 0.05 to 250 μg/mL anti-TfR mAbs A27.15 and E2.3 alone or in combination in triplicates. Cell viability was measured at days 2, 4, 6, and 8 by measuring metabolic conversion (by viable cells) of the dye 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfoo-phenyl)-2H-tetrazolium, inner salt (MTS) Cell Titer 96 AQueous One Solution Cell Proliferation Assay (Promega, Southampton, United Kingdom). In each well of a 96-well plate, MTS (20 μL) was added, and plates were incubated for 2 to 4 hours in cell culture incubator. MTS assay results were read in a 96-well format plate reader by measuring absorbance at 490 nm.

Cell viability by particle Coulter counter. Cells were seeded at 2.5 × 10^4/mL (1 mL in 24-well plates) 24 hours before experimental treatments. Cells were treated with 10 and 50 μg/mL anti-TfR mAbs A27.15 and E2.3 alone or in combination. Cell viability was measured at days 1, 2, 3, and 4. Cells were washed in PBS, trypsinized with 200 μL trypsin/EDTA, and resuspended to a final volume of 1 mL with cell medium. Cell numbers were counted with a Coulter particle size analyzer (Beckman Coulter, High Wycombe, United Kingdom).

Vascular endothelial growth factor ELISA. Vascular endothelial growth factor (VEGF) secretion into the culture medium was measured using Duoset ELISA Development Human VEGF Immunnoassay (R&D Systems, Minneapolis, MN) following the manufacturer's protocol and 3,3',5,5'-Tetramethylbenzidine Liquid Substrate System for ELISA (Sigma). VEGF ELISA assay results were read in a 96-well format plate reader by measuring absorbance at 450 nm with correction at 540 nm.

Western blotting. Cells were homogenized in lysis buffer (6.2 mol/L urea, 10% glycerol, 5 mM DTT, 1% SDS plus protease inhibitors). Whole-cell extract (40 μg) was heat denatured at 95°C, separated by 8% or 10% SDS-PAGE, and transferred to polyvinylidene difluoride membrane. Primary antibodies were mouse anti-HIF-1α mAb (BD Transduction Laboratories, Lexington, KY), mouse anti-TfR mAb (PharMingen, Oxford, United Kingdom), mouse anti-CAIX M75 mAb (a gift from Dr. J. Pastorek, Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic; ref. 19), mouse anti-PHD3 mAb (Abcam), mouse anti-BNIP3 (Sigma), and mouse anti-β-tubulin mAb (Sigma). Immunoreactivity was visualized with horseradish peroxidase–linked goat anti-mouse serum and chemiluminescence.

Cellular LIP assay. LIP in cells was measured using a modified Darbari et al. (20) and Epsteine et al. (21) protocol based on the fluorescent probe calcein. This probe binds iron rapidly, stoichiometrically, and reversibly while forming fluorescence-quenched calcein-iron complexes. For LIP assay, cells were washed with loading medium serum and bicarbonate-free 2% RPMI with 20 mM HEPES and 1 mg/mL bovine serum albumin (BSA) and incubated for 10 minutes at 37°C with loading medium containing 250 mM/L calcein-AM (Molecular Probes, Cambridge, United Kingdom), an acetoxythiochelate precursor of calcein. Once inside the cells, calcein-AM is hydrolyzed by endogenous esterase into the highly negatively charged green fluorescent calcein, the fluorescence of which is quenched on binding to iron and retained in the cytoplasm. Excess calcein-AM was washed four times with loading medium, and cells were maintained at room temperature in loading medium until use. Just before measurement, loading medium was replaced with 2 mL prewarmed HBS [20 mM HEPES, 150 mM NaCl (pH 7.3)] containing 10 mM/L glucose, and fluorescence was monitored at an excitation of 488 nm and emission of 517 nm using a fluorescence plate reader for 10 minutes to establish a stable baseline signal. Estimation of calcein-iron fluorescence was obtained by adding 100 μmol/L 2,2'-bipyridyl (BIP) in ethanol, a highly permanent and high-affinity iron chelator, and fluorescence was measured as before for 20 minutes. Ethanol alone was used as control instead of BIP. The method for assessing LIP reflecting the concentration of cellular labile iron was done by calculating the fractional increase in fluorescence signal (∆F) given as fluorescence arbitrary units elicited by the addition of BIP compared with background fluorescence and ethanol control-treated cells in a given number of cells.

CAIX flow cytometry. Mouse monoclonal anti-human CAIX antibody M75 was used for flow cytometric analysis. mAb M75 (10 μL) was added to tube containing a 50 μL aliquot of 0.5 × 10^6 to 1 × 10^6 cells. This working volume of mAb was set up from preliminary tests on cell lines. An isotype IgG (DAKO, Ely, United Kingdom) was used as a negative control. Cells and antibody were gently mixed and incubated at 4°C for 30 minutes. FITC-conjugated goat anti-mouse immunoglobulin antisera (10 μL; DAKO) was added after washing with 2 mL PBS-0.1% BSA-4 mM/L EDTA solution. After another 30-minute incubation in the dark at 4°C, cells were washed with 2 mL PBS-BSA-EDTA solution. Samples were analyzed by flow cytometry (XL Beckman, High Wycombe, United Kingdom). A typical viable cell area was gated and 1 × 10^5 events were counted. The isotype negative control was used to define the threshold of the background staining. Results were expressed as ratio between the median value of anti-CAIX mAb and isotype control stained sample.

Results

Effects of anti-TfR mAb on cell viability. Cell viability was measured by Cell Titer 96 MTS assay. Addition of anti-TfR mAb E2.3 to human breast cancer cell line MDA-468 had very little effect in reducing cell proliferation, whereas anti-TfR mAb A27.15 resulted in a moderate concentration-dependent reduction in cell proliferation following 4 days of treatment (Fig. 1A). In contrast, exposure to equal concentrations (10-250 μg/mL) of both anti-TfR mAbs together resulted in concentration-dependent synergistic reduction in cell proliferation following 4 days of treatment, with up to 80% inhibition in cell proliferation following 8 days of exposure to both anti-TfR mAbs at 250 μg/mL. IgG1 control did not inhibit cell proliferation following 8 days of treatment (Fig. 1B). Synergistic inhibition of cell proliferation was also observed when MDA-231 and MCF-7 cell lines were treated for 8 days with equal concentrations (10-250 μg/mL) of both anti-TfR mAbs together (Fig. 1C).

Effects of anti-TfR mAbs on HIF-1α, HIF-1α transcription targets, and TIR expression. To assess whether TIR transported iron contributes to PHD function and regulation of HIF-1α, VEGF, a downstream target gene of HIF-1α, was measured by ELISA by collecting cell medium from MDA-468 cells treated with anti-TfR mAbs (Fig. 2A and B). Addition of anti-TfR mAb E2.3 or A27.15 resulted in a moderate concentration-dependent induction of VEGF after 4 days. In contrast, exposure to equal concentrations (0.5-250 μg/mL) of both anti-TfR mAbs together resulted in concentration-dependent synergistic induction of VEGF after 4 days compared with theoretical combined value of VEGF pg/mL when cells were treated with A27.15 or E2.3 alone.

We next wanted to determine if anti-TfR mAbs can also up-regulate HIF-1α in normoxic MDA-468 cells (Fig. 2C). Although the iron chelator desferrioxamine is well known to do this (22), the role of the normal uptake and pools for transferrin has not been studied. Treatment of MDA-468 with 50 μg/mL anti-TfR mAb A27.15 or E2.3 had a moderate effect in increasing HIF-1α protein levels following 24 hours or 4 days of treatment, whereas equal concentration of anti-TfR mAbs A27.15 and E2.3 at 50 μg/mL together significantly up-regulated HIF-1α following 24 hours or 4 days of treatment. Levels of CAIX, PHD3, and BNIP3, downstream targets of HIF-1α in MDA-468 cells, were also higher in cells treated with combined anti-TfR mAbs compared with cells treated with A27.15 mAb or E2.3 mAb alone. Similar results were also observed when MDA-231 cell line was treated with anti-TfR mAbs (Fig. 2D). HIF-1α and BNIP were up-regulated following treatment with combined anti-TfR mAbs.

It has been shown that TIR can be up-regulated by HIF-1α via the hypoxia response element in the TIR gene (23) and...
that TfR can also be negatively regulated post-transcriptionally by intracellular iron through iron-responsive elements in the 3'-untranslated region of the TfR mRNA (8). We therefore analyzed the effect of anti-TfR mAb on TfR expression in MDA-468 cells. Anti-TfR mAb A27.15 or E2.3 (50 μg/mL) had very little effect in changing the level of TfR expression (Fig. 2C), whereas treatment with both 50 μg/mL anti-TfR mAbs A27.15 and E2.3 together down-regulated TfR expression. Similar results were also observed when MDA-231 cell line was treated with anti-TfR mAbs (Fig. 2D). TfR was down-regulated following treatment with combined anti-TfR mAbs.

**Effect of anti-TfR mAb on LIP.** Because combined anti-TfR mAbs inhibited proliferation and up-regulated HIF-1α, we tested to see if this was due to a reduction in cellular LIP. MDA-468 cells were treated with combined 50 μg/mL anti-TfR mAbs A27.15 and E2.3 in triplicates for 24 hours in six-well plates. After the addition of calcein-AM and stabilization in the fluorescence readings (usually 20 averaged time points over a 10-minute period), the control cells responded to the addition of BIP by a discrete increase in fluorescence signal ΔF = 35 due to the release of calcein from iron (Fig. 3A), showing a detectable LIP, whereas anti-TfR mAb-treated cells responded with a smaller increase in fluorescence signal ΔF = 13 (Fig. 3B), showing lower level of cellular LIP. Cells treated with ethanol as control instead of BIP only produced a very small increase in fluorescence signal. From the mean values of ΔF minus the ethanol control ΔF from four samples treated or not treated with anti-TfR mAb, the addition of anti-TfR mAbs significantly (P < 0.05, Student’s t test) reduced the cellular LIP by 60% (Fig. 3C).

**Effects of ascorbate and anti-TfR mAb on cell viability.** Because we have shown that combining anti-TfR mAbs inhibits MDA-468 breast carcinoma cell proliferation and up-regulates HIF-1α by lowering the cellular LIP, we next tested to see if ascorbate can reverse the antiproliferative effects of anti-TfR mAb. Breast cancer cell line MDA-468 cells were treated with 10 or 50 μg/mL...
anti-TfR mAbs A27.15 and E2.3 in the presence or absence of 25 μmol/L ascorbate, which was added every 24 hours due to its short half-life, for 4 days. Cell viability was measured using cell particle counter (Fig. 4A). Untreated and ascorbate-treated cells expanded by a factor of 9 and 7, respectively, after 4 days, whereas cells exposed to combined 10 or 50 μg/mL anti-TfR mAbs A27.15 and E2.3 had a reduction in cell proliferation. The addition of ascorbate did not reverse the antiproliferative effect of anti-TfR mAbs.

Effects of ascorbate and anti-TfR mAb HIF. Because ascorbate is required by PHD enzymes for full catalytic activity in normoxic cells (24), we tested to see if ascorbate can down-regulate HIF-1α in MDA-468 cells treated with combined anti-TfR mAbs. Using the same day 4 cells, ascorbate significantly blocked the up-regulation of CAIX and VEGF in cells treated with combined anti-TfR mAbs analyzed by fluorescence-activated cell sorting (FACS) and ELISA, respectively (Fig. 4B and C). This shows that ascorbate at physiologic concentrations suppressed HIF transcription targets but did not suppress the antiproliferative effect of the combined anti-TfR mAbs.

We analyzed the time course effect of ascorbate on HIF-1α in MDA-468 cells treated with anti-TfR mAbs for up to 4 days (Fig. 5A). Treatment of MDA-468 breast carcinoma cells with both anti-TfR mAbs A27.15 and E2.3 together at 50 μg/mL induced the expression of HIF-1α protein (Fig. 5A). In the presence of 25 μmol/L ascorbate, which was added every 24 hours, levels of HIF-1α induced by anti-TfR mAbs were inhibited completely at 8 hours and 24 hours, whereas on day 4 the levels of HIF-1α were suppressed but not inhibited completely.

We analyzed the expression of HIF-1α target genes by Western blotting. CAIX and BNIP3, which are both up-regulated in cells treated with anti-TfR mAbs, remained at basal levels in cells treated with anti-TfR mAb in combination with ascorbate, consistent with the effects observed on HIF-1α protein levels and data from CAIX FACS analysis and VEGF ELISA. The same effect was observed with the MDA-231 cell line (Fig. 5B). Combined mAb treatment up-regulated HIF-1α and HIF-α target gene BNIP, whereas the addition of ascorbate prevented the up-regulation of HIF-1α and HIF-α target genes by anti-TfR mAbs.
Discussion

There has been a growing interest in the potential usefulness of iron deprivation as a component of cancer therapy. There are currently three main agents in clinical studies. The first is based on gallium nitrate, which interferes with the release of iron from endocytic vesicles, the second is iron chelation, and the third treatment is based on the use of mAbs or small molecules against transferrin or the TfR (25–33). In this study, we showed that two different anti-TfR mAbs A27.15 and E.2.3 against the external domain of TfR synergistically inhibited proliferation of cultured breast tumor cells in vitro and can block the growth of s.c. MDA-231 tumors in vivo when 1.5 mg combined anti-TfR mAb (0.75 mg A27.15 and 0.75 mg E2.3) was i.p. injected daily for 15 days into BALB/c severe combined immunodeficient mice (data not shown). Both these anti-TfR mAbs and other anti-TfR mAbs have been shown previously to have synergistic antiproliferative effects on human cell lines cells in vitro and inhibit the growth and induce tumor regressions of established s.c. tumors of CCRF-CEM leukemia cells in nude mice (17, 34). Breast cancer cell lines have also been shown to express high levels of TfR compared with normal breast epithelial cells and are more sensitive to iron depletion, which leads to inhibition of cell proliferation (35–37). The synergistic inhibition of proliferation induced by two anti-TfR mAbs is likely related to the ability of the mAbs to bind to different epitopes in TfR, resulting in receptor cross-linking, and block in iron uptake, which is required for DNA synthesis (9, 38). In addition, following treatment with combined anti-TfR mAbs, expression of TfR was down-regulated or degraded as a result of TfR cross-linking, which may also contribute to iron deprivation (17, 34).

However, the new findings in our study are that blocking the uptake of iron via the TfR resulted in the up-regulation of VEGF. This was mediated through the stabilization of HIF-1α via inhibiting proline hydroxylation by PHDs as a result of reduction in the cellular LIP. Many others have shown that the iron chelator...
Desferrioxamine can also up-regulate VEGF (39), but this could be through direct removal of iron from intracellular compartments or enzymes. However, desferrioxamine is a poor iron chelator with poor antiproliferative activity and iron chelation efficacy (40). This study shows that extracellular iron transported through the TfR is used by the PHDs, showing a new and rapidly responsive physiologic pathway of HIF-1α response. Thus, the induction of erythropoietin in iron deficiency may be related to response to low iron transport rather than effects of low oxygen from anemia. In addition, there are insufficient iron stores or mechanisms to release iron to supply PHDs if the external source is removed.

Although the treatment of tumors with potent iron chelators or anti-TfR mAb results in HIF-1α stabilization and VEGF expression and could promote angiogenesis, iron depletion is well known to cause cell cycle arrest and apoptosis (41–44). Without constant supply of iron, the conversion of ribonucleotides to deoxyribonucleotides by ribonucleotide reductase is inhibited and DNA synthesis is prevented (44). Up-regulation of HIF-1α by anti-TfR or iron chelators may also induce apoptosis via induction of BNIP3 (7, 45), a proapoptotic Bcl-2 member (46). However, in an in vivo setting, up-regulation of angiogenic factors and CAIX may increase tumor vasculature and resistance to acidic stress (47).

Because ascorbate has been shown to have an effect on down-regulating the expression and activity of the HIF system (24), we decided to test if ascorbate had an effect on HIF-1α and downstream targets of HIF-1α and proliferation of cells treated with anti-TfR mAbs. Here, we showed that ascorbate at physiologic concentration down-regulated HIF-1α expression and HIF-1α transcription of VEGF, CAIX, and BNIP3 in cells treated with anti-TfR mAbs.

The mechanism of action is not completely understood. Ascorbate is thought to increase the activity of PHDs by increasing the availability of Fe(II) either free in solution or within the enzyme active site by reducing Fe(III) to Fe(II) (3, 48). In the absence of ascorbate, PHDs can rapidly catalyze the hydroxylation of proline. However, under these conditions, the enzyme becomes inactivated as the enzyme-bound Fe(II) is rapidly converted to Fe(III). Enzyme-bound Fe(III) can be reduced by ascorbate, thus reactivating the enzyme. Although ascorbate may stimulate the activity of PHDs by reducing iron from Fe(III) to Fe(II), the stimulation of oxygenase activity by ascorbate may occur through other mechanisms (e.g., by promoting completion of uncoupled cycles; ref. 49). It is believed that one role of ascorbate is to function as a surrogate-reducing substrate to rescue the enzyme in the event of uncoupled production of a ferryl [Fe(IV)] intermediate. Ascorbate may also have a role in the binding of prime substrate to the enzyme (50).

Whichever mechanism used by PHDs, our findings indicate that ascorbate has large effects on the HIF system through promotion of HIF hydroxylase activity, even when cells are starved of iron when treated with combined anti-TfR mAbs. This implies that, without ascorbate or iron, PHD enzyme activity is suboptimal, allowing stabilization of HIF-1α and up-regulation of downstream targets in normoxic conditions. More surprisingly, ascorbate did not rescue cells from antiproliferative effects of anti-TfR mAbs, which suggests separate iron pools regulating these processes or different iron thresholds between PHDs and enzymes required for cell proliferation and survival.

The proapoptotic BNIP3 in anti-TfR-treated cells were shown to be down-regulated in cells treated with ascorbate, which suggests...
that BNIP3 plays no role in apoptosis in anti-TfR mAb-treated cells. It has been reported that ascorbate can induce apoptosis in melanoma cells, which seems to be initiated by a reduction of TfR expression, resulting in a down-regulation of iron uptake followed by an induction of apoptosis (51). Here, in breast cancer cell line MDA-468, ascorbate alone did not induce cell death or down-regulate TfR expression (data not shown).

Novel iron chelators that have high permeability, such as desferrioxamin analogues, tachpyridine, O-trenox, pyridoxal isonicotinoyl hydrazone analogues, and Triapine, have antiproliferative activity and cause a marked cell cycle arrest and apoptosis in vitro and in vivo (41, 43, 44). Consideration should be given to combining these agents with ascorbate in their clinical trials. Pharmacodynamic monitoring of plasma VEGF should be possible to evaluate the effects.

In conclusion, combination of anti-TfR mAbs inhibits tumor cell proliferation and induces HIF response, which can be blocked by physiologic concentration of ascorbate. Vitamin C deficiency is common in cancer patients (52). Clinical use of anti-TfR mAbs is likely to induce the HIF response through iron depletion, and effects may be more pronounced in patients because of the vitamin C deficiency. Our demonstration of the separate iron regulation involved in the HIF and proliferation response due to iron depletion by blocking TfR suggests that administration of anti-TfR mAb combined with physiologic doses of ascorbate may be beneficial.

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