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Identification of Heme Oxygenase-1 As a Novel BCR/ABL-Dependent Survival Factor in Chronic Myeloid Leukemia

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ABSTRACT

Chronic myeloid leukemia (CML) is a stem cell disease in which BCR/ABL promotes the survival of leukemic cells. Heme oxygenase-1 (HO-1) is an inducible stress protein that catalyzes the degradation of heme and has recently been implicated in the regulation of growth and survival of various neoplastic cells. In the present study, we analyzed the expression and role of HO-1 in CML cells. As assessed by Northern and Western blot analysis as well as immunostaining, primary CML cells were found to express HO-1 mRNA and the HO-1 protein in a constitutive manner. Exposure of these cells to the BCR/ABL tyrosine kinase inhibitor STI571 resulted in decreased expression of HO-1 mRNA and protein. In addition, BCR/ABL was found to up-regulate HO-1 promoter activity, mRNA levels, and protein levels in Ba/F3 cells. To investigate the role of HO-1 for survival of primary CML cells, the HO-1 inducer hemin was used. Hemin-induced expression of HO-1 was found to protect CML cells from STI571-induced cell death. In addition, inhibition of HO-1 by zinc-(II)-deuteroporphyrin-IX-2,4-bisethyleneglycol resulted in a substantial decrease of cell viability. Furthermore, overexpression of HO-1 in the CML-derived cell line K562 was found to counteract STI571-induced apoptosis. Together, our data identify HO-1 as a novel BCR/ABL-driven survival molecule and potential target in leukemic cells in patients with CML. The pathogenetic and clinical implications of this observation remain to be elucidated.

INTRODUCTION

Chronic myelogenous leukemia (CML) is a myeloid neoplasm characterized by the Philadelphia chromosome, which is generated by the reciprocal chromosomal translocation t(9;22) (1, 2). This translocation fuses the *c-abl* gene from chromosome 9 to the *bcr* gene locus on chromosome 22 (3). The resulting *bcr/abl* oncogene encodes a M_r 210,000 oncoprotein (BCR/ABL), which displays constitutive tyrosine kinase activity and thus leads to "malignant transformation" of hematopoietic precursor cells. In particular, BCR/ABL-transformed cells exhibit enhanced proliferative capacity, altered adhesion properties, and reduced apoptosis (4, 5). Notably, BCR/ABL-dependent inhibition of apoptosis is considered to play a crucial role in growth and accumulation of leukemic cells in patients with CML.

During the past few years, a number of attempts have been made to identify critical genes and factors that regulate survival and apoptosis in CML cells (6). Apart from external factors (cytokines; Ref. 7), a number of cellular molecules have been implicated in the regulation of apoptosis in BCR/ABL-transformed cells. Likewise, depending on the cell type analyzed, members of the bcl-2 family (bcl-x_L, bcl-2, and A1) reportedly counteract apoptosis (8–11). However, little is known about the relative contribution of each of these molecules to survival of leukemic cells. In addition, some of these molecules may only be expressed in leukemic cells in a subset of patients with CML (12). Therefore, it seems of importance to further search for new antiapoptotic molecules expressed in CML cells.

Heme oxygenase-1 (HO-1) is an inducible stress protein that catalyzes the degradation of heme to carbon monoxide (CO), biliverdin, and iron (13, 14). Recently, HO-1 has been implicated in the regulation of apoptosis in various mesenchymal cells (14). Although the precise mechanisms of HO-1-dependent survival are not known, recent data suggest that antiapoptotic effects of HO-1 are mediated through heme degradation products.

Thus far, most studies analyzing the role of HO-1 as an antiapoptotic molecule have focused on endothelial cells and fibroblasts (15–19). More recent data suggest, however, that cancer cells in solid tumors can also express HO-1 (20–23). In the present study, we show that CML cells express HO-1 in a constitutive manner and that the BCR/ABL oncogene promotes HO-1 gene expression. In addition, our data suggest that HO-1 plays a role in BCR/ABL-dependent survival of CML cells. Thus, HO-1 may represent a novel BCR/ABL-dependent, antiapoptotic effecter molecule and potential target in CML.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Ton.B210-X is a growth factor-dependent, Ba/F3-derived hematopoietic cell line in which the M_r 210,000 form of BCR/ABL can conditionally be induced through the addition of doxycycline (24, 25). Ton.B210-X cells were grown in RPMI 1640 (Mediatech Cellgrow, Herndon, VA) with 10% FCS (Life Technologies, Inc., Carlsbad, CA) and 10% WEHI-3B conditioned medium (as a source of murine interleukin-3) at 37°C and 5% CO_2. For starvation, Ton.B210-X cells were cultured in the absence of interleukin-3 for ≤48 h. All experiments with Ton.B210-X cells were performed in UltraCulture medium (BioWhittaker, Walkersville, MD).

The Philadelphia chromosome-positive, CML-derived K562 cell line and BCR/ABL-transformed Ba/F3 cells (Ba/F3p210 cells) were maintained in RPMI 1640 with 10% FCS at 37°C and 5% CO_2. To inhibit BCR/ABL tyrosine kinase activity, cells were exposed to 1 μM STI571 (kindly provided by Novartis Pharma AG, Basel, Switzerland) for 16–48 h. To induce expression of HO-1, cells were incubated with hemin (10–20 μM; Sigma, St. Louis, MO) for 16–48 h at 37°C and 5% CO_2. After exposure to compounds, cells were analyzed for survival and/or subjected to Northern or Western blot analysis.

Isolation and Culture of Primary Cells. Primary leukemic cells were obtained from 15 patients with untreated chronic phase CML. Informed consent was obtained before blood donation. Peripheral blood mononuclear cells were isolated by Ficoll density centrifugation. Isolated peripheral blood mononuclear cells were cultured in RPMI 1640 with 10% FCS at 37°C and 5% CO_2.

Immunocytochemistry. Immunocytochemistry was performed on cytospin preparations of primary leukemic cells (bone marrow and peripheral blood mononuclear cells) obtained from 6 patients with chronic phase CML. Acetone-fixed slides were incubated with a polyclonal rabbit anti-HO-1 antibody (work dilution: 1:1000; Stressgen Biotechnologies Corp., Victoria, British Columbia, Canada) for 60 min. After washing, slides were incubated with a biotinylated goat antirabbit IgG (Biocarta, San Diego, CA) for 30 min and then with streptavidin-alkaline-phosphatase complex (Biocarta) for another 30 min.

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Neofuchsin (Nichirei, Tokyo, Japan) was used as chromogen. Slides were counterstained in Mayer's hemalaun. In control experiments, slides were preincubated with a HO-1 blocking peptide (Stressgen) before antibody exposure.

HO-1 Reporter Gene Assay. To determine HO-1 promoter activity, the HO-1-luc construct (Ref. 26; a kind gift of Shigeru Takahashi, Scripps Research Institute, La Jolla, CA; 10 μg) was transfected together with a pCMV-βGAL construct (10 μg) by electroporation (0.35 kV, 960 μF, Gene Pulser; Bio-Rad, Hercules, CA) into Ton.B210-X cells (1×10^7 in 800 μl). Thereafter, cells were either maintained in the presence or absence of doxycycline (1 μg/ml) for 18 h in UltraCulture medium. Cells were then harvested, and the pellets were resuspended in lysis buffer (Promega, Madison, WI). Lysates (30 μl) were incubated with 100 μl of luciferase assay buffer [25 mM glycylglycine, 15 mM potassium phosphate (pH 7.8), 15 mM $MgSO_4$, 4 mM EGTA, 2 mM ATP, and 1 mM DTT] and 100 μl of D-luciferin (0.3 mg/ml; PharMingen, San Diego, CA). Luciferase activity was determined by an automated luminometer (Wallac 1420 multilabel counter; Perkin-Elmer, Turku, Finland). Plasmid pCMV-βGAL (Invitrogen, Carlsbad, CA) was used as a reporter for transfection efficiency. For determination of βGAL-activity, we used the Invitrogen βGAL Assay Kit (Invitrogen). HO-1 reporter gene activity was given as a ratio of luciferase activity:βGAL-activity.

Northern Blot Analysis. Total RNA was extracted from Ton.B210-X cells or primary CML-derived peripheral blood mononuclear cells using Trizol (Life Technologies) according to the manufacturer's instructions. Northern blotting was performed essentially as described (25). In brief, 20 μg of total RNA were size fractionated on 1% formaldehyde-agarose gels and transferred to nylon membranes (Hybond N; Amersham, Aylesbury, United Kingdom) as described by Chomczynski (27). Membranes were hybridized in rapid-hyb buffer (Amersham). Hybridization was performed with ^{32}P-labeled cDNAs specific for HO-1 and β-actin. Primers for PCR amplification of probes were as follows: murine HO-1: 5′-CCTCACTGGCAGGAAATCAT-3′ (forward) and 5′-TCTCTGCAGGGGCAGTATCT-3′ (reverse); murine β-actin: 5′-GACGGCCAGGTCATCACTAT-3′ (forward) and 5′-AGGGAGACCAAAGCCTTCAT-3′ (reverse); human HO-1: 5′-ATGGAGCGTCCGCAACCCGA-3′ (forward) and 5′-GCATAAAGCCCTACAGCAAC-3′ (reverse); human β-actin: 5′-ATGGATGATGATATCGCCGCG-3′ (forward) and 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC-3′ (reverse). Labeling was performed using the Megaprime kit (Amersham). Blots were washed in

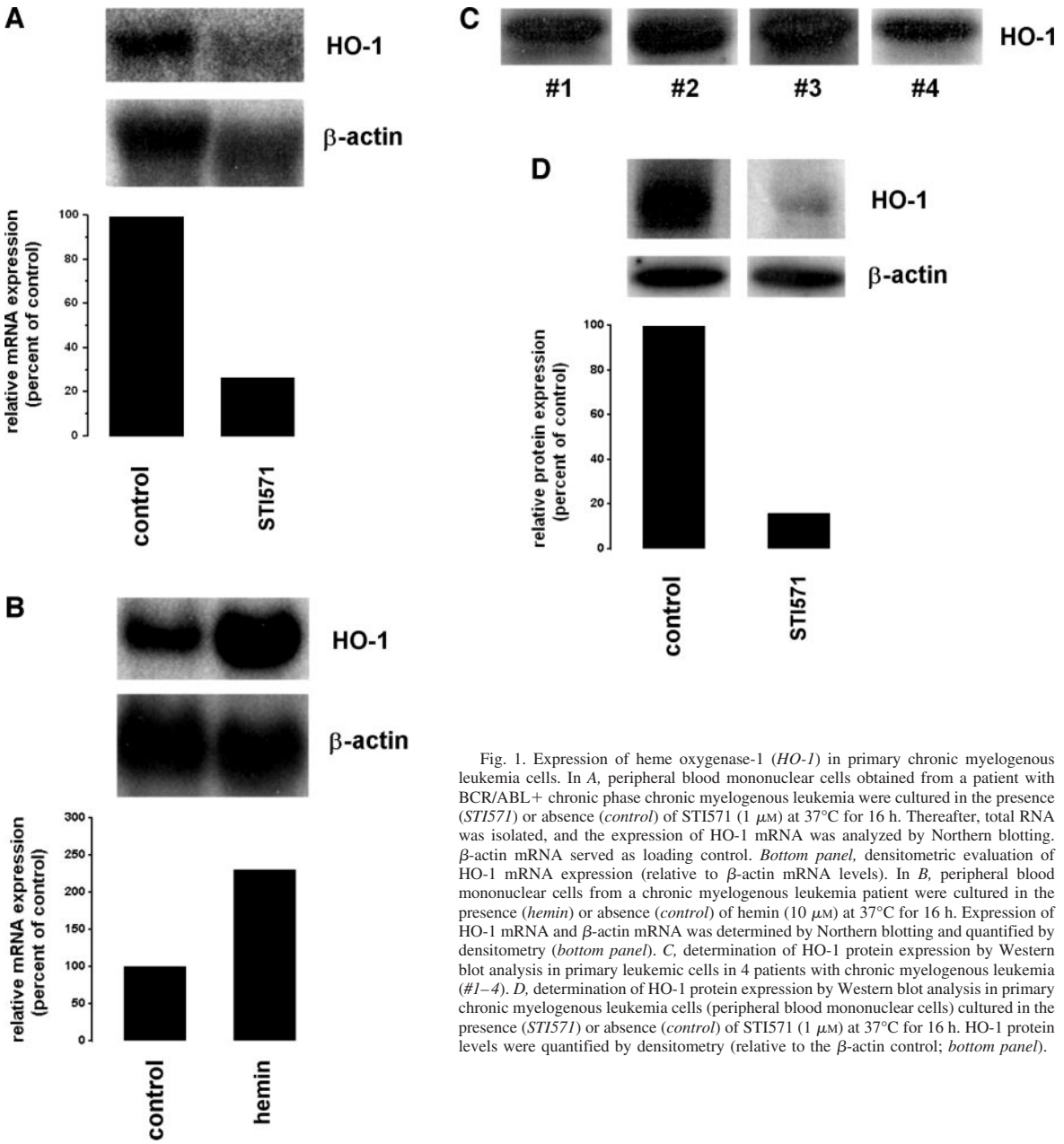

Fig. 1. Expression of heme oxygenase-1 (*HO-1*) in primary chronic myelogenous leukemia cells. In *A,* peripheral blood mononuclear cells obtained from a patient with BCR/ABL+ chronic phase chronic myelogenous leukemia were cultured in the presence (*STI571*) or absence (*control*) of STI571 (1 μM) at 37°C for 16 h. Thereafter, total RNA was isolated, and the expression of HO-1 mRNA was analyzed by Northern blotting. β-actin mRNA served as loading control. *Bottom panel,* densitometric evaluation of HO-1 mRNA expression (relative to β-actin mRNA levels). In *B,* peripheral blood mononuclear cells from a chronic myelogenous leukemia patient were cultured in the presence (*hemin*) or absence (*control*) of hemin (10 μM) at 37°C for 16 h. Expression of HO-1 mRNA and β-actin mRNA was determined by Northern blotting and quantified by densitometry (*bottom panel*). *C,* determination of HO-1 protein expression by Western blot analysis in primary leukemic cells in 4 patients with chronic myelogenous leukemia (*#1–4*). *D,* determination of HO-1 protein expression by Western blot analysis in primary chronic myelogenous leukemia cells (peripheral blood mononuclear cells) cultured in the presence (*STI571*) or absence (*control*) of STI571 (1 μM) at 37°C for 16 h. HO-1 protein levels were quantified by densitometry (relative to the β-actin control; *bottom panel*).

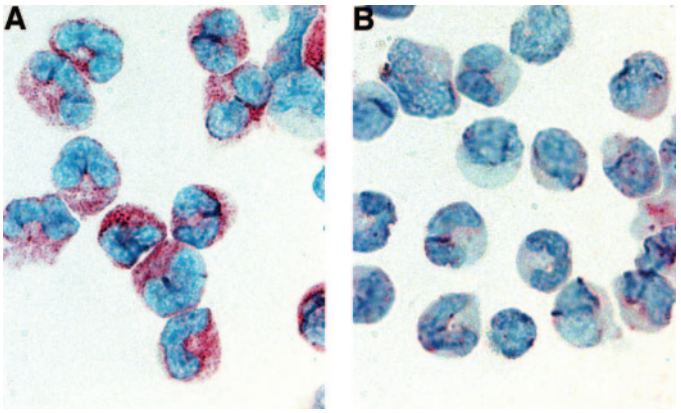

Fig. 2. Detection of heme oxygenase-1 in primary chronic myelogenous leukemia cells by immunocytochemistry. *A,* bone marrow mononuclear cells obtained from a patient with BCR/ABL+ chronic phase chronic myelogenous leukemia were spun on a cytospin slide and stained with an antibody against heme oxygenase-1. *B,* preincubation with a heme oxygenase-1 blocking peptide before antibody staining (described in *A*), abrogated heme oxygenase-1 immunoreactivtiy of primary chronic myelogenous leukemia cells.

0.2 × SSC [1 × SSC = 150 mM NaCl and 15 mM sodium citrate (pH 7.0)] with 0.1% SDS at 42°C for 2 × 30 min and at 62°C for another 30 min. Bound radioactivity was visualized by exposure to Biomax MS film (Eastman Kodak, Rochester, NY) at −80°C using intensifying screens (Kodak). mRNA expression levels were quantified by densitometry of autoradiograms using the E.A.S.Y. Win32 software (Herolab, Wiesloch, Germany).

Preparation of Cell Lysates and Western Blotting. Cells were washed in 0.9% NaCl and then lysed by distilled water and freeze thawing. Cellular proteins were separated under reducing conditions by SDS-PAGE and then transferred to a nitrocellulose membrane (Protran, Schleicher & Schuell Bioscience, Keene, NH) in buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol at 4°C. The membrane was blocked for 1 h in 3% BSA. After incubation with a rabbit anti-HO-1 polyclonal antibody (Stressgen), the membrane was exposed to goat antirabbit IgG (Amersham). To confirm equal loading, membranes were reprobed with a rabbit antiactin antibody (Sigma). Chemoluminescence was detected by exposure to Biomax MS film (Kodak).

Transient Expression of HO-1 and Cell Sorting. K562 cells (5×10^6 cells in 800 μl) were transfected by electroporation (960 microfarads, 200 V; Gene-Pulser Bio-Rad) with 15 μg of pCDNA3-HO-1 (kindly provided by Shigeki Shibahara, Tohoku University School of Medicine, Sendai, Japan; Ref. 28) or empty pCDNA3 vector and with 10 μg of a plasmid containing enhanced green fluorescent protein (Clontech, Palo Alto, CA). After electroporation (24 h), enhanced green fluorescent protein-expressing cells were sorted on a high-speed cell sorter (FACSAria; Becton Dickinson, Heidelberg, Germany). After sorting, cells were cultured in RPMI 1640 plus 10% FCS at 37°C and 5% CO_2 for 24 h. Then, cells were split and cultured in the absence or presence of STI571 (500 nM) for another 24 h at 37°C and 5% CO_2. Thereafter, the percentage of apoptotic cells was assessed by annexin V-staining. For this purpose, cells were washed in PBS and then incubated with annexin V-APC (Alexis Biochemicals, Lausen, Switzerland) in binding buffer containing HEPES [10 mM (pH 7.4)], NaCl (140 mM), and $CaCl_2$ (2.5 mM). Cells were then washed and analyzed by flow cytometry (FACSCalibur; Becton Dickinson).

Treatment of Cells with CO, Biliverdin, and Desferrioxamine (DFX). K562 cells were grown in a humidified air atmosphere containing either 5% CO_2 without CO or 5% CO_2 together with 1% CO (kindly provided by AGA Linde Health Care, Lidingö, Sweden) as described by Otterbein *et al.* (29) for 48 h. During the first 3 h, cells were kept in CO-containing or control atmosphere without additional compounds. Then, various combinations of STI571 (1 μM), biliverdin (100 μM; Porphyrin Products, Logan, UT), DFX (100 μM; Novartis), or hemin (20 μM) were added. After 48 h, the percentage of apoptotic cells was determined.

Determination of Cell Viability and Apoptosis. To investigate the function of HO-1 in BCR/ABL-dependent survival of leukemic cells, primary CML cells (patients in chronic phase CML, $n = 3$), K562, and Ba/F3p210 cells were analyzed. K562 and Ba/F3p210 cells were incubated with STI571 (1 μM) in the presence or absence of hemin (20 μM) at 37°C and 5% CO_2 for 48 h. Primary CML cells were incubated with STI571 (1 μM) in the presence or absence of hemin (20 μM; Ref. 28) at 37°C and 5% CO_2 for 3 weeks. In these experiments, STI571 was replaced every 48 h, and hemin was replaced every 96 h.

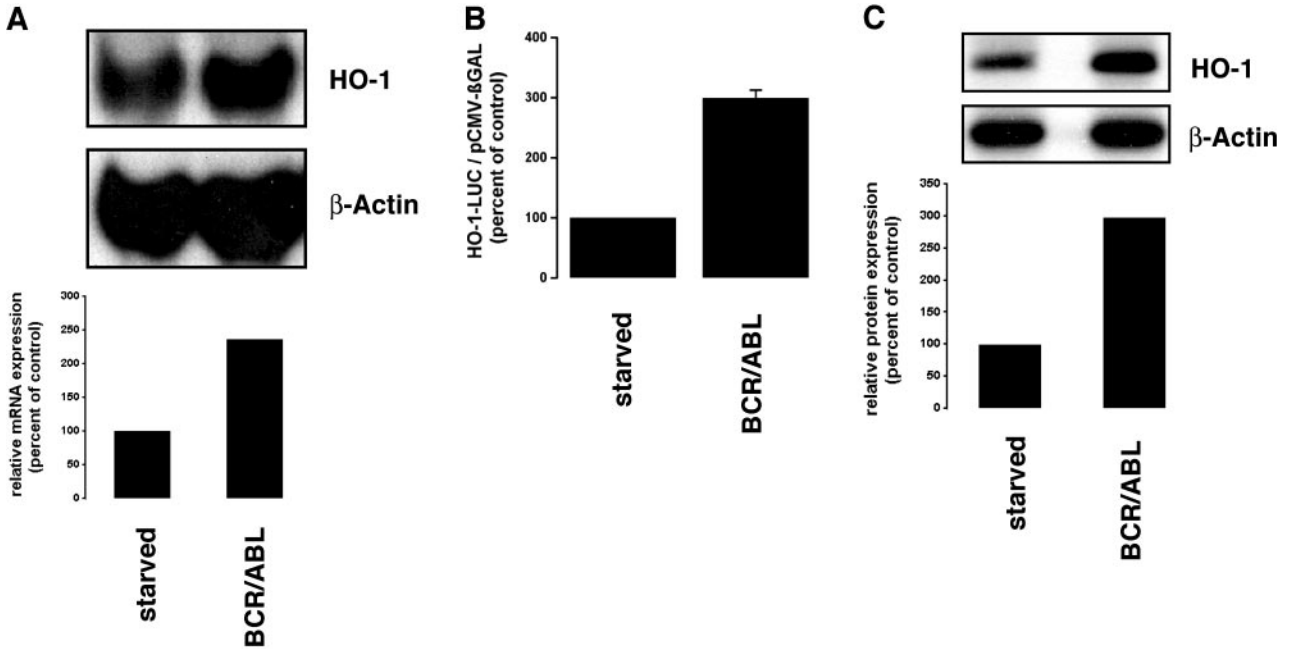

Fig. 3. BCR/ABL-induced heme oxygenase-1 (*HO-1*) gene expression in Ton.B210-X cells. *A,* Northern blot analysis. Ton.B210-X cells were maintained without interleukin-3 for 16 h (37°C) in the presence (*BCR/ABL*) or absence (*starved*) of doxycycline. Expression of HO-1 mRNA was detected by Northern blotting. The β-actin loading control is also shown. A densitometric evaluation of HO-1 mRNA levels is shown in the *bottom panel*. *B,* HO-1 promoter activity. Ton.B210-X cells were transfected with a HO-1 promoter construct (*HO-1-LUC*) and pCMV-βGal and grown in the presence (*BCR/ABL*) or absence (*starved*) of doxycycline for 16 h. After incubation, cells were harvested and assayed for the expression of luciferase and βGal activities. Luciferase activity was reported as the ratio HO-1-luc:pCMV-βGal and expressed as the percentage of control (starved cells). Results represent the means ± SD of three independent experiments. *C,* Western blot analysis of Ton.B210-X cells. Cells were maintained without interleukin-3 for 48 h in the presence (*BCR/ABL*) or absence (*starved*) of doxycycline and subjected to Western blotting using antibodies against HO-1 and β-actin. A densitometric evaluation of protein levels is shown in the *bottom panel*.

In addition, CML cells were cultured in the presence or absence of the HO-1 inhibitor zinc-(II)-deuteroporphyrin-IX-2,4-bisethyleneglycol (ZnDPPIX; Ref. 30) at 37°C and 5% CO_2 for 3 weeks. ZnDPPIX was applied at a concentration of 10 μM and replaced every 96 h. For determination of cell viability, cells were washed in PBS and stained with propidium iodide (1 μg/ml) before being subjected to flow cytometry (FACScan; Becton Dickinson). Cells were defined as being nonviable (propidium positive) when shifting above autofluorescence levels of unstained cells. Cell viability was also determined by the trypan blue exclusion test. The percentage of apoptotic cells was quantified on cytospin preparations (Wright-Giemsa stained). Apoptosis was defined according to conventional cytomorphological criteria (31).

RESULTS

Primary CML Cells Express HO-1 mRNA and the HO-1 Protein in a Constitutive Manner. Recent data suggest that HO-1 is expressed in neoplastic cells in patients with various solid tumors (20, 21). In the present study, we found that HO-1 mRNA is constitutively expressed in primary leukemic cells in chronic phase CML (Fig. 1*A*). As assessed by Northern blotting, HO-1 mRNA expression was detectable in all patients analyzed ($n = 10$). The BCR/ABL tyrosine kinase inhibitor STI571 (1 μM) counteracted HO-1 mRNA expression in leukemic cells (Fig. 1*A*), suggesting a possible role for BCR/ABL in HO-1 gene expression. Hemin, a potent inducer of HO-1, was found to up-regulate expression of HO-1 mRNA in primary CML cells (Fig. 1*B*). As determined by Western blotting, the HO-1 protein was found to be expressed in primary CML cells in 4 of 4 patients analyzed (Fig. 1*C*). Treatment of cells with STI571 (1 μM) for 16 h resulted in a substantial decrease in expression of the HO-1 protein (Fig. 1*D*). We next examined HO-1 protein expression in CML cells by immunocytochemistry. As expected, the HO-1 protein was detectable in leukemic cells by immunostaining in all CML patients ($n = 6$) analyzed (Fig. 2*A*). Preincubation of CML cells with a HO-1 blocking peptide abrogated HO-1 immunoreactivity (Fig. 2*B*), confirming the specificity of the antibody reaction. All in all, these data show that primary CML cells express HO-1 mRNA and the HO-1 protein in a constitutive manner and that HO-1 expression in these cells is sensitive to treatment with STI571, suggesting a potential role for BCR/ABL in HO-1 expression.

BCR/ABL Transcriptionally Activates the *HO-1* Gene and Increases HO-1 mRNA and Protein Levels in Ton.B210-X Cells. To investigate whether BCR/ABL directly induces HO-1 gene expression, Ton.B210-X cells grown in the presence of doxycycline were compared with starved (interleukin-3 deprived) cells. As assessed by Northern blotting, doxycycline-induced expression of BCR/ABL led to an ~2–3-fold increase in expression of HO-1 mRNA in these cells (Fig. 3*A*). We next asked whether induction of HO-1 by BCR/ABL would depend on transcriptional activation of the *HO-1* gene. For this purpose, Ton.B210-X cells were transfected with a HO-1 reporter gene construct. After transfection, cells were split and either maintained in the presence or absence of doxycycline. As shown in Fig. 3*B,* BCR/ABL significantly increased HO-1 promoter activity compared with starved cells. Similarly, the expression of BCR/ABL augmented HO-1 protein levels as shown by Western blot analysis (Fig. 3*C*). These data show that the BCR/ABL oncogene promotes HO-1 expression through transcriptional activation of the HO-1 promoter in Ton.B210-X cells.

HO-1 Counteracts STI571-Induced Apoptosis in BCR/ABL-Transformed Cells. To investigate the role of HO-1 for survival of BCR/ABL-transformed cells, experiments using the CML-derived cell line K562, as well as Ba/F3p210 cells, were performed. As shown in Fig. 4*A,* induction of HO-1 by hemin was found to counteract STI571-induced cell death in K562 cells. In particular, exposure of K562 cells to STI571 (1 μM) for 48 h resulted in a substantial decrease of cell viability, and addition of hemin (20 μM) rescued these cells from STI571-induced cell death (Fig. 4*A*). Corresponding results were obtained with BCR/ABL-transformed Ba/F3 cells (data not shown). To show that hemin indeed leads to induction of HO-1 in STI571-treated cells, Northern blot experiments were performed. In these experiments, hemin-treated cells were found to express HO-1 mRNA in the absence as well as presence of STI571 in Ba/F3p210 cells (Fig. 4*B*), and the same was found to hold true for K562 cells (data not

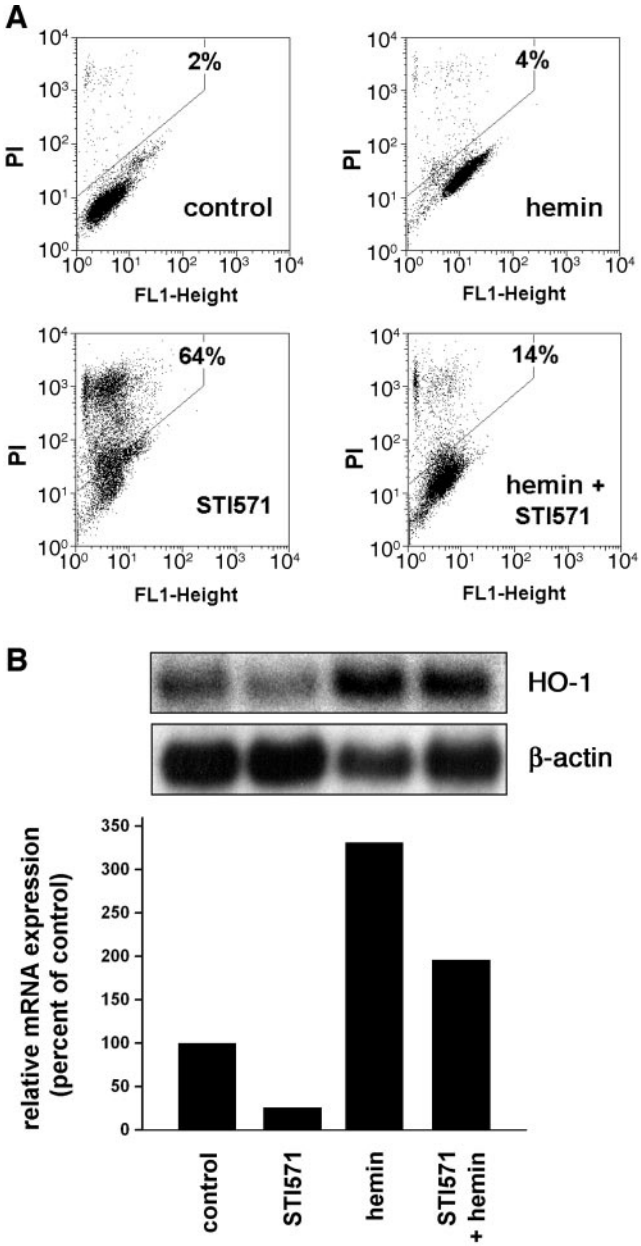

Fig. 4. Induction of heme oxygenase-1 (*HO-1*) by hemin counteracts apoptosis in BCR/ABL-transformed cells. In *A,* K562 cells were incubated in control medium (*control*), hemin (20 μM), STI571 (1 μM), and hemin (20 μM) plus STI571 (1 μM) at 37°C for 48 h. After incubation, cells were stained with propidium iodide (*PI*; 1 μg/ml) and analyzed by flow cytometry. A cutoff gate based on autofluorescence levels of unstained cells was used to discriminate viable from nonviable (*PI* positive) cells. The percentages of *PI*-positive cells under various conditions are depicted. The figure shows one of three independent experiments with almost identical results. In *B,* Ba/F3p210 cells were incubated in control medium (*control*), hemin (20 μM), STI571 (1 μM), and hemin (20 μM) plus STI571 (1 μM) at 37°C for 16 h. Thereafter, RNA was isolated and subjected to Northern blotting. Expression of HO-1 mRNA was detected using a cDNA probe specific for human HO-1. Expression of β-actin is shown as a loading control. A densitometric evaluation of HO-1 mRNA expression levels is shown in the *bottom panel*.

shown). To provide additional evidence for the protective effect of HO-1 (against STI571-induced apoptosis), K562 cells were transfected with a vector containing the HO-1 cDNA. As expected, the STI571-induced apoptosis of K562 cells was markedly reduced in HO-1-transfected K562 cells compared with cells transfected with the empty control vector (Fig. 5). All in all, these data suggest that HO-1 plays an important role as an antiapoptotic molecule in BCR/ABL-transformed cells.

Functional Role of HO-1 in Primary CML Cells. To demonstrate functional significance of HO-1 expression in primary CML cells, ZnDPPIX, an inhibitor of HO-1 activity, was applied. Treatment of primary CML cells with ZnDPPIX (10 μM) for 21 days resulted in a substantial decrease in cell viability and an increased rate of apoptosis compared with untreated cells (Fig. 6*A*). To investigate the role of HO-1 as a survival-promoting factor in CML cells in the context of BCR/ABL, primary CML cells were treated with the BCR/ABL tyrosine kinase inhibitor STI571 (1 μM) in the presence or absence of hemin (20 μM). As expected, STI571 reduced the viability of CML cells in a time-dependent manner (Fig. 6*B*). However, induction of HO-1 by hemin was found to protect these cells from STI571-dependent cell death (Fig. 6*B*). Moreover, treatment with hemin was found to counteract STI571-induced apoptosis in these cells (Fig. 6*C*).

Role of CO, Biliverdin, and Iron for Survival of CML Cells. To investigate the mechanism(s) of HO-1-dependent survival of CML cells, K562 cells were exposed to the heme degradation products CO and biliverdin. To mimic the effects of HO-1 on cellular iron levels, the iron chelator DFX was also applied in these experiments. Exposure of cells to either CO (1%), biliverdin (100 μM), or DFX (100 μM) alone showed no effect on STI571-induced apoptosis in K562 cells (data not shown). However, in the presence of CO, DFX and biliverdin were found to partially rescue K562 cells from apoptosis induced by STI571 (Fig. 7). Simultaneous exposure to CO, biliverdin, and DFX did not further enhance survival of STI571-treated K562 cells compared with the combinations CO plus DFX or CO plus biliverdin (Fig. 7). Together, these data suggest that heme degradation products may play a role in HO-1-mediated survival of CML cells. However, because we could not mimic the effect of hemin by applying CO, biliverdin, and DFX, it seems likely that other mechanisms are also involved in the survival-promoting function of HO-1 in CML cells.

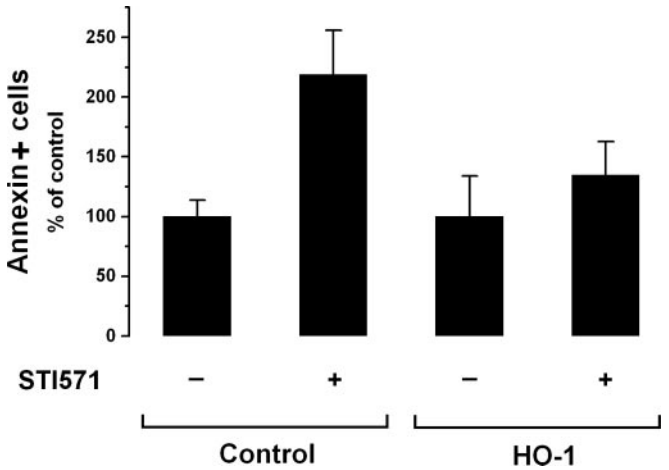

Fig. 5. Expression of heme oxygenase-1 (*HO-1*) protects K562 cells from STI571-induced apoptosis. K562 cells were transfected with pCDNA3-HO-1 (HO-1) or empty pCDNA3 vector (*Control*). Then, cells were split and cultured in the absence or presence of STI571 (500 nM) for 24 h. Thereafter, the percentage of apoptotic cells was assessed by annexin V staining. Results represent the means ± SD of three independent experiments.

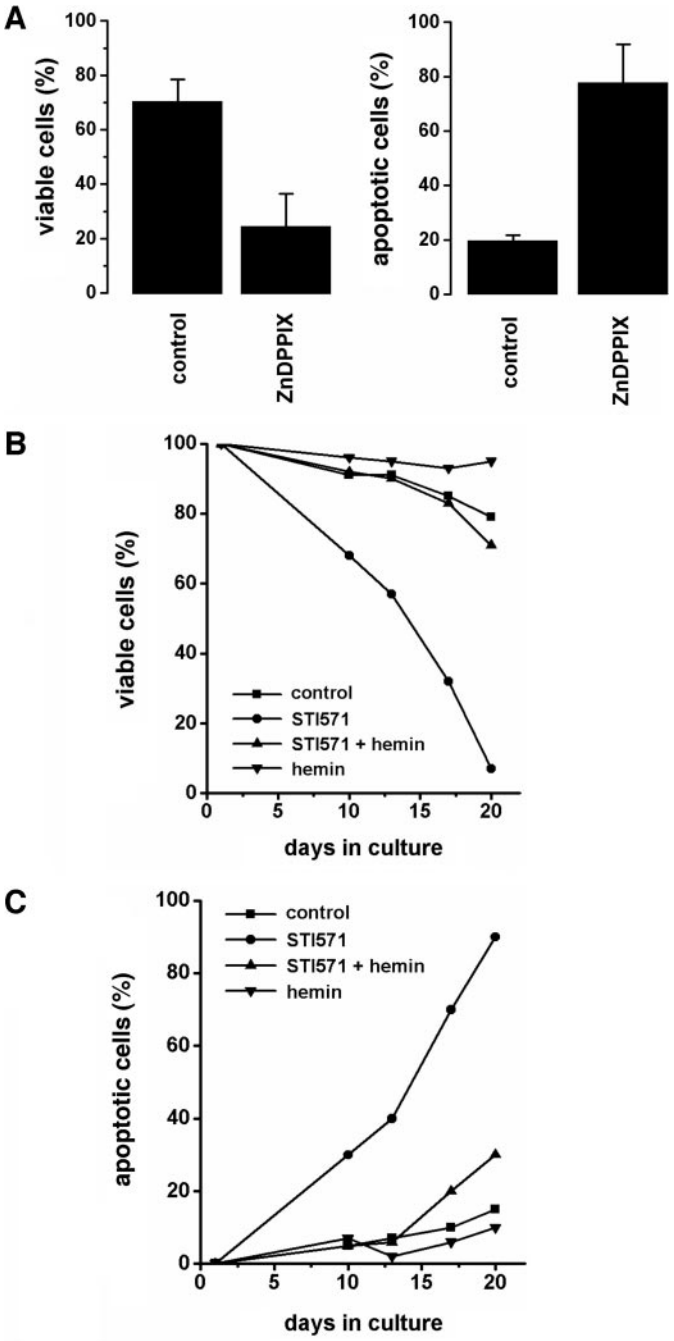

Fig. 6. Role of heme oxygenase-1 for survival of primary chronic myelogenous leukemia cells. In *A,* chronic myelogenous leukemia cells obtained from patients with untreated chronic phase chronic myelogenous leukemia were maintained in control medium (*control*) or the presence of the heme oxygenase-1 inhibitor zinc-(II)-deuteroporphyrin-IX-2,4-bisethyleneglycol (*ZnDPPIX*; 10 μM). After 21 days, cells were harvested, and cell viability was determined by the trypan blue exclusion (*left panel*). The percentage of apoptotic cells was quantified in cytospin preparations (*right panel*). Results represent the means ± SD of three independent experiments (cells from three different chronic myelogenous leukemia patients). In *B,* primary leukemic cells isolated from a patient with untreated chronic phase chronic myelogenous leukemia were maintained in control medium (*control*), STI571 (1 μM), STI571 (1 μM) plus hemin (20 μM), or hemin (20 μM) for 21 days. After various time periods (as indicated), cells were harvested and examined for cell viability using the trypan blue exclusion test. In addition, the percentage of apoptotic cells was quantified (*C*).

DISCUSSION

A number of studies have shown that HO-1 plays a role as an antiapoptotic molecule in endothelial cells and other non-neoplastic mesenchymal cells (15, 19, 32). More recent data suggest that HO-1

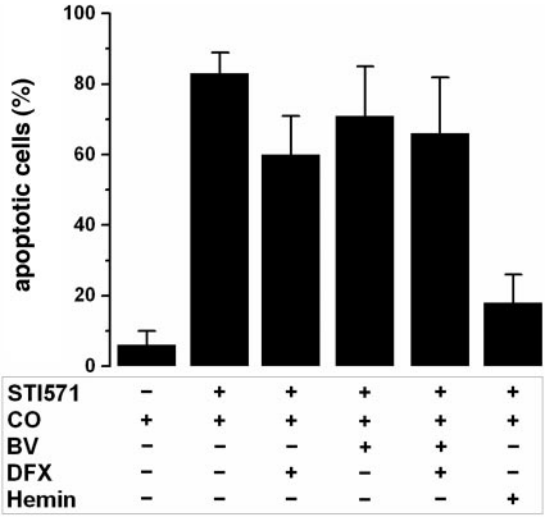

Fig. 7. Effects of carbon monoxide (*CO*), biliverdin, and desferrioxamine (*DFX*) on STI571-induced apoptosis in K562 cells. K562 cells were cultured in the presence or absence of STI571 (1 μM), CO (1%), biliverdin (100 μM), DFX (100 μM), or hemin (20 μM) as indicated for 48 h. Thereafter, the percentage of apoptotic cells was quantified on cytospin preparations. Results represent the means ± SD of three independent experiments.

may also contribute to enhanced survival of neoplastic cells in various tumors (20–23). In the current study, we show that primary leukemic cells in patients with CML express HO-1 and that the disease-related oncoprotein BCR/ABL up-regulates expression of HO-1 in Ba/F3 cells. Moreover, our data suggest that HO-1 plays an important role in BCR/ABL-dependent survival of CML cells and that targeting of HO-1 in leukemic cells is associated with decreased cell survival.

The observation that leukemic cells in patients with CML express HO-1 in a constitutive manner is a remarkable finding that is consistent with expression of HO-1 in solid tumors (20–23). By contrast, most normal cells and tissues are considered to express only low amounts of (or to lack) HO-1 unless exposed to certain (stress inducing) stimuli (13). On the basis of this notion, we were interested to know whether the disease-related oncoprotein BCR/ABL plays a role in the constitutive expression of HO-1 in CML cells. Supporting this hypothesis, the BCR/ABL tyrosine kinase inhibitor STI571 was found to down-regulate expression of HO-1 in primary CML cells. Moreover, we found that BCR/ABL induces expression of HO-1 in Ba/F3 cells. To the best of our knowledge, this is the first study showing that a leukemia-related oncogene promotes HO-1 gene expression. Whether HO-1 is also expressed in other leukemias and/or nonhematopoietic malignancies in an oncogene-dependent manner remains at present unknown. In this regard, it is of interest that also oncogenic Ras was found to induce HO-1 gene expression in Ba/F3 cells.[3] Thus, it may be tempting to speculate that oncogene-dependent expression of HO-1 is a more general phenomenon that may occur in various (myeloid) neoplasms.

A number of recent studies have implicated HO-1 as an antiapoptotic effector molecule in mesenchymal cells exposed to oxidative stress (15, 19). To address the question as to whether HO-1 also acts as a survival factor in leukemic cells, HO-1 was overexpressed in BCR/ABL-transformed cells by two independent approaches. In a first step, K562 and Ba/F3 cells were exposed to hemin, resulting in up-regulation of HO-1. These hemin-exposed (HO-1 overexpressing) cells were found to be far less susceptible to STI571-dependent cell killing compared with CO cells. To directly show that HO-1 exerts a protective effect against STI571, K562 cells were transiently transfected with the HO-1 cDNA, and again, HO-1 was found to counteract STI571-induced apoptosis in K562 cells.

The above observations prompted us to ask whether HO-1 can be used as a novel molecular target in primary CML cells. To address this issue, a specific inhibitor of HO-1, ZnDPPIX (30), was applied and found to decrease cell viability and increase apoptosis in primary CML cells. In this regard, it is of interest, that a water-soluble pegylated zinc protoporphyrin (PEG-ZnPP) has recently been developed and found to act as an antineoplastic agent in experimental solid tumors *in vivo* (33). Whether a similar approach can be developed far enough in leukemias to enter clinical trials in the future remains at present unknown. Likewise, it may be of interest to test whether HO-1 expression plays a functional role in STI571-resistant CML cells and whether combination therapy using STI571 and a HO-1-targeting drug would counteract the development or overcome resistance against STI571.

A number of studies have shown that HO-1 exerts antiapoptotic effects through generation of heme degradation products, including biliverdin, iron, and CO (19, 23, 32). We therefore asked whether these degradation products are involved in HO-1-dependent survival of CML cells. The results of our study show that various combinations of biliverdin, CO, and DFX counteract STI571-induced cell death in CML cells. However, no significant effect was seen when these substances were applied as single agents. Moreover, even when used in combination, the STI571-induced apoptosis could only in part be reverted by these compounds contrasting their effects on endothelial cells and fibroblasts (15, 16). This discrepancy may have several explanations. A reasonable explanation would be that additional HO-1-dependent mechanisms (apart from CO, biliverdin, and iron) play a role in cell survival in CML cells. Thus, the mechanisms underlying HO-1-induced survival in neoplastic (leukemic) cells may differ from that contributing to HO-1-dependent survival in non-neoplastic cells. Another reason for the different effects of CO, biliverdin, and DFX in CML cells as opposed to other cell types may be the different stimuli used to induce apoptosis. The possibility that the divergent result was caused by differences in the methodology applied (*i.e.,* in CO exposure) could be excluded by the demonstration that CO inhibited the lipopolysaccharide-induced production of tumor necrosis factor-α in RAW 264.7 mouse macrophage cells in the same way as described by Otterbein *et al.* (Ref. 29; data not shown).

In summary, our data show that primary CML cells express HO-1 in a constitutive manner and that expression of this gene is associated with increased survival of leukemic cells. Moreover, our data show that targeting of HO-1 in CML cells is associated with reduced survival and increase in apoptosis. Whether these observations will have clinical implications in the future remains at present unknown.

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