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Hepatocyte Growth Factor Receptor c-MET Is Associated with FAS and When Activated Enhances Drug-induced Apoptosis in Pediatric B Acute Lymphoblastic Leukemia with TEL-AML1 Translocation*

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Expression of c-MET, the HGF (hepatocyte growth factor) tyrosine kinase receptor, was investigated in pediatric B-acute lymphoblastic leukemia (ALL) patients. c-MET was found to be expressed in normal B cells and in B-ALL patients with the t(12;21) TEL-AML1 translocation, but it is not expressed in the most part of B-ALL without the t(12;21). We also found that c-MET, related to proliferation and protection from apoptosis, is associated with the pro-apoptotic protein FAS in TEL-AML1 B-ALL cells and in normal B lymphocytes. The possible role of this protein complex in drug-induced apoptosis was thus investigated in REH TEL-AML1 B-ALL cell line. REH cells prestimulated with HGF and treated with doxorubicin had shown a higher apoptotic rate than non-HGF-prestimulated ones ($p = 0.03$). REH cells stimulated with IL-3 and treated with doxorubicin did not undergo apoptosis more than nonstimulated cells, demonstrating that increased proliferation in itself is not directly related to the higher apoptotic sensitivity observed with HGF stimulation. These results indicate that c-MET activation enhances specifically FAS-mediated apoptosis in TEL-AML1 ALL cells and, considering that the c-MET/FAS complex is present only in normal B lymphocytes and in TEL-AML1 leukemias, this implies that it may have an important contribution in cellular homeostasis and in high sensitivity of TEL-AML1 ALL to chemotherapeutic regimens.

Acute lymphoblastic leukemia (ALL)[2] is the most common form of childhood cancer and is the primary cause of cancer-related mortality in children (1). ALL is a disorder in which failure of differentiation, overproliferation, and a defective apoptosis in a clonal cell compartment result in accumulation of aberrant cells. Modifications in key cellular genes, such as growth factor, death receptor, and transcription factor genes, underpin the basis of leukemogenesis. These genetic modifications could result in a series of acquired defects that allow the developing cancer cell to grow unchecked and resist to drug cytotoxic effects (2).

The tyrosine kinase c-MET is the high affinity receptor for HGF/SF (hepatocyte growth factor/scatter factor), a multifunctional cytokine with pleiotropic effects (3, 4). After HGF binding, c-MET undergoes autophosphorylation at tyrosine residues Tyr^{1234} and Tyr^{1235}, creating a multifunctional docking site for several proteins including Grb2, PI3K, PLCγ, SHP-2, and STAT3. Signaling via this pathway has been shown to affect a wide range of biological activities including cell motility, growth, proliferation, and protection from apoptosis. Thus, uncontrolled HGF/c-MET signaling triggers a unique biological program conferring metastatic properties to cancer cells (5–8). In a wide variety of epithelial cancers, alterations of c-MET activity because of mutations, overexpression or coexpression with HGF had been well-described (for a review see Ref. 9).

Remarkably, coordinated control of the HGF/c-MET pathway is also important in vertebrate development. Mouse knockout experiments result in embryonic death because of defects in epithelial cell-based organs and nervous system formation (10). c-MET has been detected also in hematopoietic cells such as human CD34+ hemopoietic progenitor cells (11), and it seems to play an important role in regulation of antigen-specific B-cell differentiation (12). Moreover, the HGF/c-MET signaling pathway was observed to be involved in development and progression of hematological neoplasias including Burkitt lymphomas

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[2] The abbreviations used are: ALL, acute lymphoblastic leukemia; HGF/SF, hepatocyte growth factor/scatter factor; PI3K, phosphatidylinositol-3′-OH kinase; PLCγ, phospholipase Cγ; SHP-2, Src homology domain 2 protein-tyrosine phosphatase; STAT3, signal transducer and activator of transcription 3; CALL, common acute lymphoblastic leukemia; FCS, fetal calf serum; PBS, phosphate-buffered saline; IL-3, interleukin 3; PI, propidium iodide; RQ-PCR, real-time quantitative polymerase chain reaction; Gus-B, β-glucuronidase; IP, immunoprecipitation; WB, Western blot; Fas-L, Fas-ligand; FADD, Fas-associated death domain; DISC, death-inducing signaling complex.

(13), Hodgkin lymphomas (14, 15), multiple myeloma (16), acute myeloid leukemias (17), and T-cell acute lymphoblastic leukemias (18).

Although it is well described that deregulation of the c-MET/HGF pathway results in unchecked overproliferation, an antiproliferative effect of HGF had been reported on melanoma and hepatocellular carcinoma cell lines, proposing an additive antitumor effect for this growth factor (19). A marked inhibition of cell growth after HGF subministration was observed also in other epithelial cancer cell types (20–23), and pretreatment with HGF in ovarian cancer cell lines was observed to increase cell sensitivity to drug-induced apoptosis (24). Thus, the HGF/c-MET signaling pathway can play opposite roles on cell survival and apoptosis in cancer. However, little is known about the molecular mechanisms underlying this dual effect. HGF/c-MET may elicit these different cellular responses depending on cell type, cell environment, and protein interactions. It has been proposed that c-MET could influence apoptosis by interacting with death receptors: in some epithelial cancer cells c-MET directly binds to the pro-apoptotic protein FAS (25, 26), suggesting that this association might have an effect on the apoptotic activity of FAS.

Here we investigate c-MET expression and its functional role in childhood B-ALL. We report that c-*Met* is expressed in B acute leukemias as well as in normal B lymphocytes. c-*Met* expression is higher in children affected by TEL-AML1 (also called ETV6/RUNX1) ALL, the subtype of childhood leukemia that very well responds to chemotherapy (27–31), than in children without this translocation, who have a less favorable prognosis. We demonstrate for the first time that in human TEL-AML1 leukemias, in REH TEL-AML1 cell line, and in normal B cells, c-MET forms a protein complex with FAS. We report also that activated c-MET, cooperating with FAS, has pro-apoptotic effects in drug-treated TEL-AML1 ALL cells. These results represent a new element concerning the role of c-MET in pediatric ALL.

EXPERIMENTAL PROCEDURES

Patients and Healthy Controls—Bone marrow samples from 99 children with newly diagnosed precursor B-ALL were analyzed at the Hematology-Oncology Laboratory of the Department of Pediatrics, University of Padova. The whole blood blast percentage for all samples was between 74 and 96%. Diagnosis was made according to standard cytomorphology, cytochemistry, and immunophenotypic criteria (32).

Bone marrow mononuclear cells from patients and healthy donors were separated by Ficoll-Hypaque technique (Amersham Biosciences). In healthy donor specimens, B cells were isolated by positive selection using Miltenyi CD19 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the isolated CD19+ B cells was over 95%, as determined by flow cytometric analysis.

PCR analysis performed for standard diagnostic procedures showed that 21 of 99 pediatric samples were positive for the chromosomal translocation t(12;21) TEL-AML1.

Cell Lines—Human leukemia cell lines REH and 697, and human hepatocellular carcinoma cell line HEP-G2 were purchased from DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The REH cell line derives from B-ALL with t(12;21) TEL-AML1 translocation, and the 697 cell line from CALL B-ALL with t(1;19). HEP-G2 cell line was used as a positive control for c-MET and FAS expression and association (26). Leukemia cell lines were cultured in RPMI 1640 (Biochrom AG, Berlin, Germany) with 10% FCS and HEP-G2 in Dulbecco's modified Eagle's medium (Sigma) with 10% FCS, penicillin (100 units/ml) and streptomycin (100 μg/ml), and maintained at 37 °C in a humidified atmosphere with 5% CO_2.

RNA Isolation and cDNA Synthesis—Total RNA was extracted from human specimens and cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. The quality of extracted RNA was verified using the Agilent Bioanalyzer 2100 and the Agilent RNA 6000 Nano Assay kit (Agilent Technologies, Palo Alto, CA), following the manufacturer's instructions. RNA concentrations were spectrophotometrically quantitated.

1 μg of total RNA was reverse-transcribed by oligo dT-primer and Superscript II reverse transcriptase (Invitrogen). RNA was primed for 10 min at 70 °C and subsequently subjected to reverse transcription for 10 min at 25 °C and 30 min at 42 °C, followed by heating at 99.9 °C for 5 min, in a total volume of 20 μl.

Real-time Quantitative PCR—c-Met and *Fas* mRNA expression levels were quantified with SYBR Green Real-Time Quantitative PCR, using the housekeeping gene *Gus-B* as reference. Reactions were performed on an ABI Prism 7700 SDS (Applied Biosystems, Foster City, CA). Primers used in this study were designed using Primer Express Software (Applied Biosystems) to amplify sequences that contain introns, so that spliced mRNA-derived signals could be distinguished from any contaminating genomic DNA-derived signals by their length: c-*Met*-forward 5′-ttggataggcttgtaagtgccc-3′, c-*Met*-reverse 5′-tactgcacttgtcggcatgaa-3′, *Fas*-forward 5′-tcacttcggaggattgctcaa-3′, *Fas*-reverse 5′-gggcattaacacttttggacg-3′, *Gus-B*-forward 5′-gaaaatatgtggttggagagctcatt-3′, *Gus-B*-reverse 5′-cggagtgaagatccccttttta-3′. To exclude contamination of unspecific PCR products, melting curve analysis was always applied to final PCR products after the cycling protocol.

Reactions were performed in a total volume of 25 μl, containing 2 μl of cDNA, 12.5 μl of SYBR Green PCR Master mix 2X (Applied Biosystems), and 300 nM forward and reverse primers. For each experiment, a serial dilution of cDNA derived from HEP-G2 cells was amplified in parallel to design the calibration curve. The expression levels of the target genes c-*Met* and *Fas* and the reference gene *Gus-B* were determined from the calibration curve using the following equation: Amount = 10^((Ct-intercept)/slope).

The target gene amount was then divided by the reference gene amount to obtain a normalized target value. The normalized target genes expressions were also calculated for healthy CD19+ B cells (calibrator). Each of the specimen-normalized target gene value was then divided by the calibrator-normalized target gene value to generate the final relative expression.

HGF and IL-3 Stimulation, Proliferation, and Pharmacological Assays—REH cell line was starved in 1% FCS for 18 h and then stimulated with 100 ng/ml of HGF (Sigma) or 20 ng/ml of

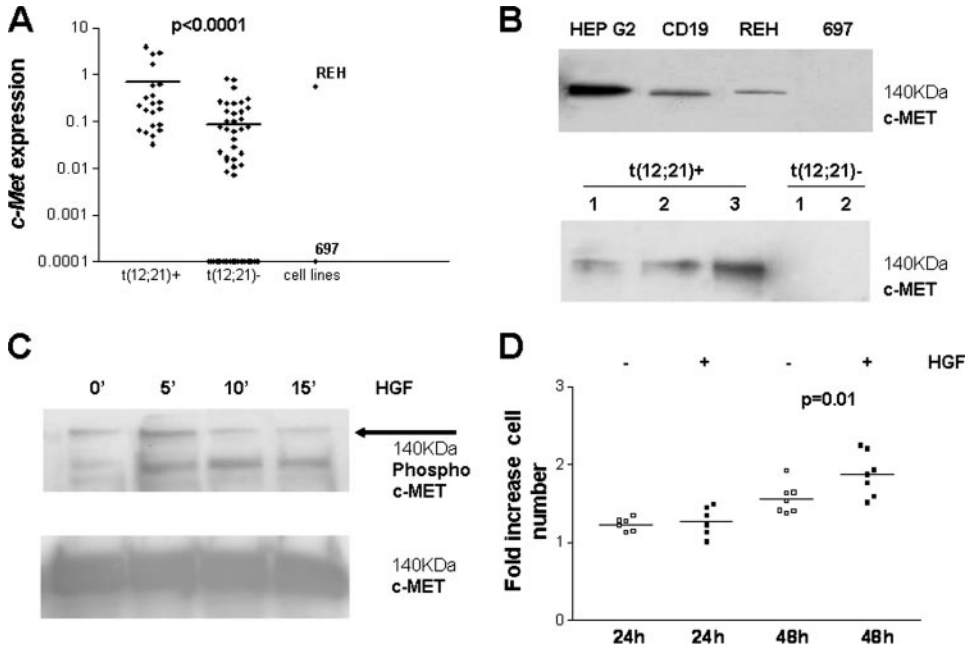

FIGURE 1. **Expression and activation of c-MET.** *A*, differential c-*Met* expression in patients with and without the t(12;21) TEL-AML1 translocation and in REH and 697 cell lines, calculated relatively to healthy CD19+ B cells (value 1 on *y*-axis, designated as calibrator), as determined by SYBR Green RQ-PCR. TEL-AML1 patients: mean 0.714, S.D. 0.247. Non TEL-AML1 patients: mean 0.084, S.D. 0.022. Mann Whitney test, $p < 0.0001$. *B*, expression of c-MET protein in cell lines (*upper gel*) (HEP-G2 cell line positive control, CD19 healthy B cells, REH TEL-AML1 B-ALL cell line, 697 t(1;19) B-ALL cell line, growing in 10% FCS medium), and in 3 patients with TEL-AML1 and 2 patients non-TEL-AML1 taken as example (*lower gel*), analyzed by Western blot with anti-c-MET-specific antibody. *C*, phosphorylation of c-MET in REH cell line after treatment with HGF. Cells were serum-starved for 18 h and then treated with HGF (30 ng/ml) for 5, 10, and 15 min. Lysates were subjected to immunoprecipitation with protein A-Sepharose beads and specific anti-c-MET antibody, and subsequently analyzed by Western blot with specific anti-phospho-c-MET antibody (IP anti c-MET, WB anti-phospho-c-MET). Membrane was stripped and reprobed with anti-c-MET antibody to control equal loading. *D*, increase (paired 2-tailed Student's *t* test, $p = 0.01$) of cell proliferation in REH cells after treatment with HGF. Cells were starved for 18 h and subsequently treated with (■) or without (□) HGF (100 ng/ml) for 24 and 48 h. Cells were microscopically counted, and cell number counts were normalized to the cell number just before HGF stimulation.

IL-3 (PeproTech Inc, Rocky Hill, NJ). Aliquots of cell suspensions were microscopically counted (diluted 1:3 with Trypan Blue) just before stimulation and after 24 h and 48 h. Apoptosis was then induced by washing cells and resuspending them in fresh medium, with or without HGF or IL-3, supplemented or not with doxorubicin (Amersham Biosciences) at 0.05 μg/ml (only in HGF-stimulated cells) and 0.1 μg/ml for 24 h. To analyze the apoptotic rate, the Annexin-V-FLUOS staining kit (Roche, Basel, Switzerland) was used following the manufacturer's instructions. Samples were analyzed by flow cytometric analysis (Cytomics FC500, Beckman Coulter, Fullerton, CA). The same HGF stimulation was performed on 697 cell line, and apoptosis was tested after 24 h of 0.5 μg/ml doxorubicin treatment.

After 24 h of doxorubicin treatment we also measured FAS and FAS-ligand expression in REH cells with a cytofluorimetric assay. We incubated 300,000 cells with 5 μl of primary antibody (anti-FAS PE-conjugated or anti FAS-L biotin-conjugated) for 15 min and, if FAS-L biotin-conjugated antibody was used, this was followed by a second incubation with 10 μl of streptavidin-PE for another 15 min. Samples were analyzed with a Cytomics FC500 (Beckman Coulter). Proteins were extracted before and after stimulation with HGF or IL-3 and from doxorubicin-treated and untreated samples.

Antibodies—The following antibodies were used for immunoprecipitation (IP), Western blot (WB), and for apoptosis assays at the concentrations reported in parentheses. Primary antibodies: anti-c-MET p140 and anti-FAS (Santa Cruz Biotechnology, Santa Cruz, CA) (IP: 2 μg/mg protein; WB: 1:500); caspase-8 (BD PharMingen, San Diego, CA) (WB: 1:400); anti-phospho-MET (Upstate Biotechnology, Lake Placid, NY) (WB: 1:400); anti α-tubulin (Sigma) (WB: 1:500). Secondary antibodies for WB: anti-rabbit and anti-mouse horseradish peroxidase-conjugated (1:10000; Sigma). The following antibodies were used for cytofluorimetric assays: anti-FAS (BD Pharmingen), anti-FAS-Ligand biotin-conjugate (BD Pharmingen), Streptavidin-PE-conjugated (Caltag Laboratories, Burlingame, CA).

Immunoprecipitation, Immunoblotting, and Reprobing of Membranes—Proteins were extracted from normal growing cells, cells treated with HGF or IL-3 alone, and with HGF or IL-3 and doxorubicin. Cells were washed twice with ice-cold PBS and lysed on ice for 20 min with lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl pH8, 150 mM NaCl, 5 mM EDTA, 150 mM $Na_4P_2O_7$, 10 mM NaF, 0.4 mM Na_3VO_4) supplemented with a complete mix of protease inhibitors (Complete Mini, Roche Applied Science). Cell lysates were then cleared by centrifugation at 13,000 × *g* for 10 min, and supernatants were collected and assayed for protein concentration with the Bradford protein assay method (Bio-Rad).

2 mg of protein were incubated with protein A- or protein G-Sepharose beads (Sigma) and immunoprecipitated with the appropriate antibody overnight at 4 °C. Proteins bound to the beads were washed three times with wash buffer (1% Nonidet P-40, 50 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA, 150 mM $Na_4P_2O_7$, 10 mM NaF, 0.4 mM Na_3VO_4) and then eluted by boiling samples in 2× Laemmli buffer (Tris-HCl 62.5 mM, pH 6.8, glycerol 10%, SDS 0.2%, bromphenol blue 0.00125%) for 5 min at 95 °C.

Total cell lysates and immunoprecipitated proteins were analyzed by SDS-PAGE under reducing conditions, and transferred to a nitrocellulose sheet (Hybond P, Amersham Biosciences) following standard methods. Primary antibodies were incubated overnight at 4 °C at the concentrations reported above. After three washes with PBS with Tween (T-PBS), membranes were incubated for 45 min at room temperature with the appropriate secondary antibody. The immunoreactivity was determined by an enhanced chemilu-

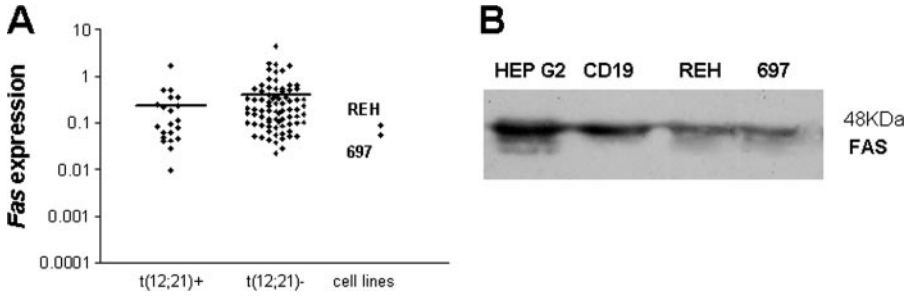

FIGURE 2. **Expression of FAS.** *A, Fas* expression in patients with and without the TEL-AML1 translocation and in REH and 697 cell lines, calculated relatively to healthy CD19+ B cells (value 1 on *y*-axis, designated as calibrator), as determined by SYBR Green RQ-PCR. TEL-AML1 patients: mean 0.238, S.D. 0.08. Non-TEL-AML1 patients: mean 0.4, S.D. 0.07. *B*, expression of FAS protein in cell lines (HEP-G2 cell line positive control, CD19 healthy B cells, REH TEL-AML1 B-ALL cell line, 697 t (1;19) B-ALL cell line) growing in 10% FCS medium, analyzed by Western blot using specific anti-FAS antibody.

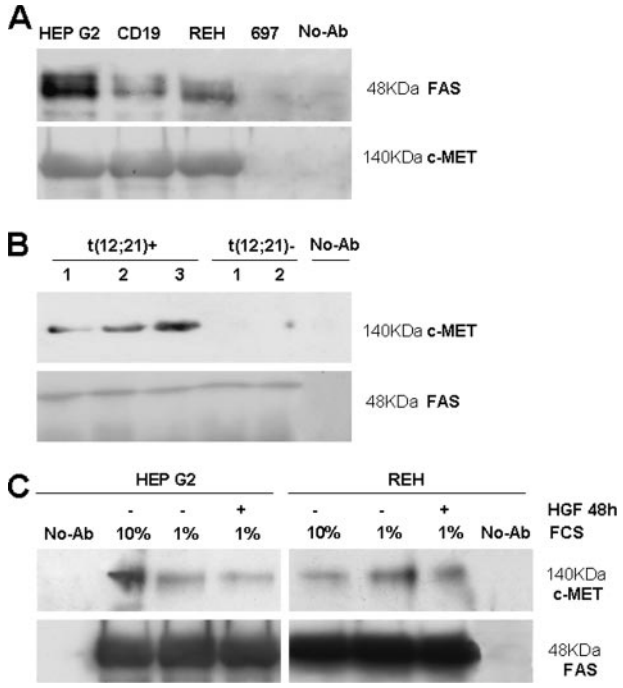

FIGURE 3. **c-MET and FAS co-precipitation in patient samples and cell lines.** *A*, co-precipitation of c-MET and FAS in cell lines. Lysates of cells growing in 10% FCS medium were immunoprecipitated with specific anti-c-MET antibody and analyzed by Western blot with specific anti-FAS antibody (IP anti-c-MET, WB anti-FAS). HEP-G2 cell line is the positive control. The lane *No-Ab* refers to samples where no primary antibody was added to cell lysates. *B*, co-precipitation of c-MET and FAS in patient samples, 3 with TEL-AML1 and 2 without TEL-AML1 taken as example. Cell lysates were immunoprecipitated with specific anti-FAS antibody and analyzed by Western blot with specific anti-c-MET antibody (IP anti-FAS, WB anti-c-MET). The lane No-Ab refers to samples where no primary antibody was added to cell lysates. *C*, co-precipitation of c-MET and FAS in HEP-G2 (positive control) and REH cell lines in standard growing conditions (10% FCS), serum-starved for 18 h (1% FCS) and after HGF stimulation (100 ng/ml, 48 h). All membranes were stripped and reprobed with anti-c-MET antibody or anti-FAS antibody to control equal protein loading. The *lanes No-Ab* refer to samples where no primary antibody was added to cell lysates.

minescent reaction (Super Signal, Pierce). For stripping of membranes, the ReBlot WB Recycling Kit (Chemicon International, Temecula, CA) was used according to the manufacturer's instructions. A negative control, consisting of samples where no primary antibody was added to cell lysate (No-Ab), was performed to detect nonspecific adherence of proteins to the beads.

Densitometric Analysis—Caspase-8 Western blot images were acquired with an Epson 3200 scanner and the relative bands were analyzed by Scion Image software (Scion Corporation, Frederick, MA). The intensity of the bands was normalized to the intensity of the bands corresponding to the α-tubulin protein. Control cells (−HGF, +doxorubicin or −IL-3, +doxorubicin) ratio was set as one.

Statistical Analysis—Statistical comparison was performed using GraphPad Prism 4 (GraphPad Software, San Diego, CA). Generally, p values for comparison of gene expression levels were based on the unpaired 2-tailed Student's t test, and proliferation rates on the paired 2-tailed Student's t test. Moreover, to compare c-*Met* expression in TEL-AML1 and non TEL-AML1 patients, p values were obtained by Mann Whitney test. Finally, to compare apoptosis rates, Wilcoxon signed-rank test was used to calculate p values.

RESULTS

c-Met Is Differentially Expressed in B-ALL Patients and Cell Lines—To investigate *c-Met* expression in precursor B-ALL pediatric patients, we tested 99 patients, 2 B-ALL cell lines and CD19+ healthy B cells by SYBR Green Real-Time Quantitative PCR (RQ-PCR). We compared patients and cell lines expression values with CD19+ healthy B cells values.

We observed a variable c-*Met* expression among analyzed patients (Fig. 1*A*): 47 patients did not express any c-*Met* mRNA, the others expressed c-*Met* at variable levels compared with normal CD19+ B cells. Thus, we looked for differences (*e.g.* chromosomal translocations, WBC count, etc.) among these patients, and we observed that it is possible to distinguish two different groups on the basis of c-*Met* expression and t(12;21) TEL-AML1 translocation. In non TEL-AML1 patients, c-*Met* is expressed in only 39.7% (31/78) of cases, and its expression is always lower compared with healthy CD19+ B cells. In TEL-AML1 patients, c-*Met* is expressed in 100% (21/21) of cases, and its expression levels are similar to those observed in normal CD19+ B cells. Thus, the expression of c-*Met* is significantly different between the TEL-AML1 and the non TEL-AML1 group of patients (Mann Whitney test, $p < 0.0001$).

The level of c-*Met* expression in the REH TEL-AML1 B-ALL cell line is similar to that detected in both TEL-AML1 B-ALL and healthy CD19+ B cells. In the 697 cell line, c-*Met* is not expressed as in the majority of the non TEL-AML1 patients.

To analyze c-MET protein expression, we performed IP/WB experiments in CD19+ healthy B cells, in patient samples, and in REH and 697 cell lines. As shown in Fig. 1*B*, upper gel, REH and CD19+ B cells express c-MET protein. In Fig. 1*B*, lower gel, is shown an example of c-MET expression in B-ALL samples, 3 with and 2 without the TEL-AML1 translocation. TEL-AML1 patients express the c-MET protein concordant with RNA expression data.

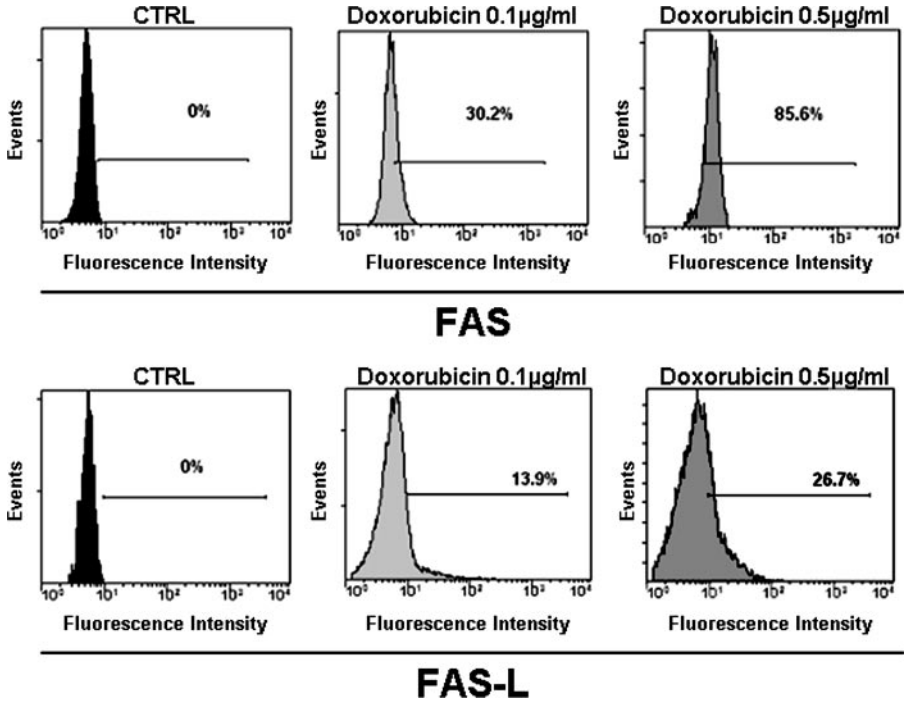

FIGURE 4. **Increase of FAS and FAS-L expression after doxorubicin treatment in REH cells.** After 18 h in serum-starved conditions, we treated REH cells for 24 h with doxorubicin at 0.1 and 0.5 μg/ml. Cells were incubated with 5 μl of anti-FAS PE-conjugated antibody or anti FAS-L biotin-conjugated antibody for 15 min. If FAS-L biotin-conjugated antibody was used, we after incubated the cells with 10 μl of streptavidin-PE for another 15 min. *Upper panel*, increase of FAS expression in a dose-dependent manner after doxorubicin treatment (30.2% of FAS-positive cells at 0.1 μg/ml of doxorubicin, and 85.6% at 0.5 μg/ml of doxorubicin). *Lower panel*, increase of FAS-L expression in a dose-dependent manner after doxorubicin treatment (13.9% of FAS-L-positive cells at 0.1 μg/ml of doxorubicin, and 26.7% at 0.5 μg/ml of doxorubicin).

This is the first observation of c-*Met* and c-MET expression in pediatric B-ALL cells. Therefore we explored its activity in the cell line harboring the TEL-AML1 translocation.

HGF Can Activate c-MET in REH Cells—To test the ability of HGF to activate c-MET, we stimulated REH cell line with HGF (30 ng/ml, for 5, 10, and 15 min) and then tested c-MET phosphorylation. As shown in Fig. 1*C*, in REH cells maximum c-MET phosphorylation occurs within 5 min after HGF stimulation. HGF is thus able to bind and activate c-MET.

We then stimulated REH cells with HGF (100 ng/ml) to test whether the HGF/c-MET pathway is active in these cells. We compared proliferation rates between HGF treated and untreated cells. As shown in Fig. 1*D*, after 24 h there was no difference in proliferation between HGF-stimulated and non-stimulated cells. After 48 h, the difference in proliferation rate is significant between nonstimulated and stimulated cells (paired 2-tailed Student's *t* test, $p = 0.01$). This result indicates that the HGF/c-MET pathway is active in these cells, and that it needs more than 24 h to increase cell proliferation.

Fas Is Underexpressed in B-ALL Patients and Cell Lines—We tested *Fas* expression levels in pediatric B-ALL patients and in B-lymphocytic cell lines by SYBR Green RQ-PCR, comparing their expression values with that in healthy CD19+ B cells.

As shown in Fig. 2*A*, in all patients *Fas* is slightly underexpressed relative to healthy B cells, with no statistically significant difference between the two groups of patients (unpaired 2-tailed Student's *t* test, $p = 0.25$). REH and 697 cell lines show expression values of *Fas* similar to that in patients. The underexpression of *Fas* in leukemic B cells relative to healthy CD19+ B cells had been previously observed (33, 34). To confirm FAS protein expression we performed IP/WB experiments with cell lines and CD19+ B cells. CD19+, REH, and 697 cells express FAS (Fig. 2*B*).

c-MET and FAS Co-precipitation—The co-precipitation of c-MET and FAS in breast neoplastic cells and in hepatoblastoma cells was observed, and this suggests that this association might have an effect on the apoptotic activity of FAS (25, 26). So we questioned whether differences in c-MET expression might have a regulatory role in FAS function. Initially we investigated whether c-MET and FAS co-precipitate in leukemia-derived cells that express both c-MET and FAS.

We show after IP anti-c-MET and WB anti-FAS that FAS co-precipitates with c-MET in REH cells and in healthy CD19+ B cells, while no co-precipitation is present in 697 cell line (Fig. 3*A*). We also investigated the co-precipitation in patient samples, and, as shown in Fig. 3*B*, the c-MET-FAS complex was observed only in TEL-AML1 patient samples, demonstrating that this association is present not only *in vitro* but also *in vivo*.

The co-precipitate remains stable in REH cells after 48 h of starvation in 1% FCS (Fig. 3*C*). To investigate if c-MET activation could disrupt the c-MET/FAS complex, we stimulated the cells for 48 h with HGF. In conditions of c-MET activation, the HGF receptor remains associated with FAS, suggesting that this complex is constitutively present in these cells (Fig. 3*C*).

We demonstrated for the first time the association of c-MET and FAS in normal B lymphocytes, in pediatric B-ALL samples and in a TEL-AML1 B-ALL cell line in starving, stimulating, and standard growth conditions. This suggests that it might be a functional interaction between these two proteins.

HGF Pretreatment Significantly Increases Apoptosis Induced by Doxorubicin in REH TEL-AML1 Cells—To understand if FAS activity, and therefore FAS-mediated apoptosis, could be affected by c-MET activation, we treated REH cells with doxorubicin (35–39) after 48 h of HGF treatment.

First, to confirm the activation of the FAS-mediated apoptosis pathway after doxorubicin treatment, we analyzed FAS and FAS-L expression (40, 41) after 24 h of treatment both with 0.1 and 0.5 μg/ml of doxorubicin in REH cells. As shown in Fig. 4, FAS and FAS-L increase their expression in a dose-dependent manner, confirming the activation of the FAS pathway in REH cells following doxorubicin treatment.

As shown in Fig. 5*A*, cells prestimulated with HGF are more sensitive to doxorubicin, when given at 0.1 μg/ml concentra-

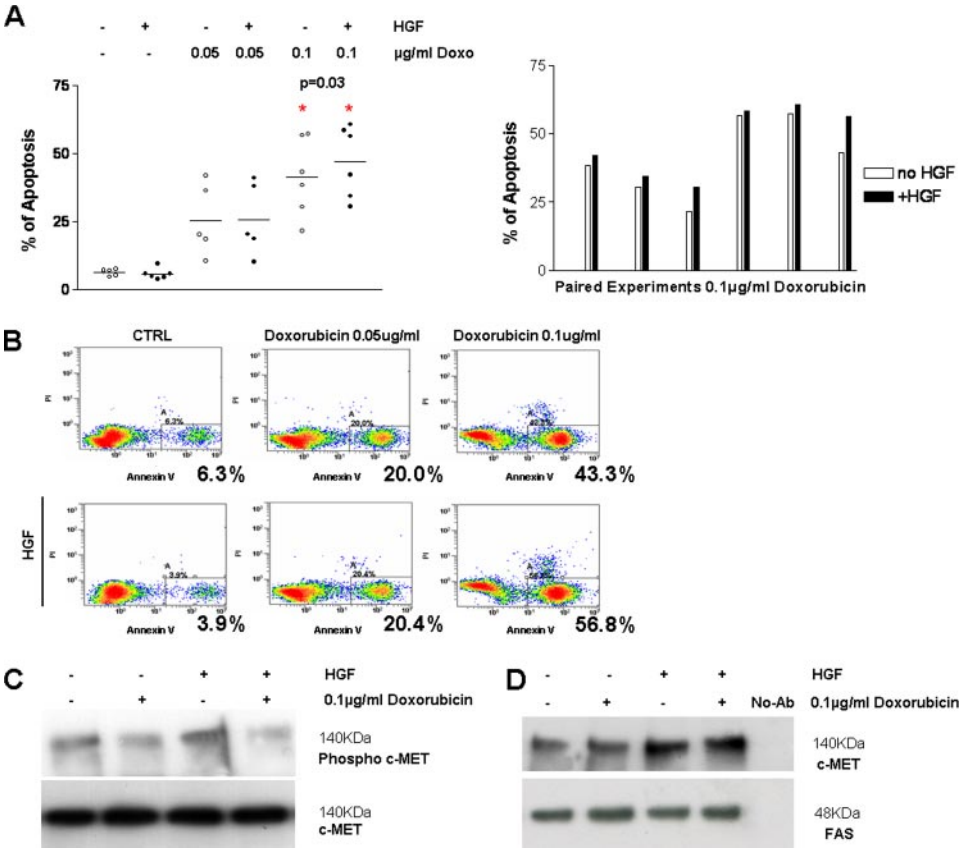

FIGURE 5. **Increase of doxorubicin-induced apoptosis after HGF treatment.** *A*, increase of apoptosis induced by doxorubicin treatment after HGF stimulation in REH TEL-AML1 cell line. Percentage of apoptosis was measured by cytofluorimetric analysis using Annexin V and PI staining. *Left panel*, where indicated cells were exposed for 24 h to doxorubicin at the reported concentrations and pretreated with HGF (100 ng/ml) for 48 h. When doxorubicin was given at a concentration of 0.1 μg/ml, apoptosis is increased more in HGF-pretreated cells (●) than in the not pretreated ones (○) (Wilcoxon test, $p = 0.03$). *Right panel*, set of the six paired experiments at 0.1 μg/ml of doxorubicin is *highlighted* in the clustered column graph. *White columns* represent the percent of apoptosis in non-HGF-prestimulated cells, *black columns* represent the percent of apoptosis in HGF-stimulated cells. *B*, cytofluorimetric example. Apoptotic cells are in the *lower part* of each plot, on the *right side* (Annexin V-positive and PI-negative). Percentage of apoptotic cells is *highlighted* near each plot. *C*, phosphorylation of c-MET in REH cell line after, where indicated, stimulation with HGF (100 ng/ml, 72 h) and treatment with doxorubicin (0.1 μg/ml, 24 h). Lysates were subjected to immunoprecipitation with specific anti-c-MET antibody, and subsequently analyzed by Western blot with specific anti-phospho-c-MET antibody (IP anti-c-MET, WB anti-phospho-c-MET). Membrane was stripped and reprobed with anti-c-MET antibody to control equal loading. *D*, co-precipitation of c-MET and FAS is stable at all different treatment conditions in REH cell line. Lysates of cells, where indicated stimulated with HGF (100 ng/ml, 72 h) and treated with doxorubicin (0.1 μg/ml, 24 h), were immunoprecipitated with specific anti-FAS antibody and analyzed by Western blot with specific anti-c-MET antibody (IP anti-FAS, WB anti-c-MET). The *lane No-Ab* refers to samples where no primary antibody was added to cell lysates.

tion, than cells non-prestimulated (Wilcoxon signed-rank test, $p = 0.03$). HGF did not induce apoptosis itself, as shown in column 2 of Fig. 5*A*, but rather caused marked sensitization of TEL-AML1 cells to subsequent treatment with doxorubicin. We show in Fig. 5*B* an example of cytofluorimetric result.

We also tested phosphorylation status of c-MET after treatment with doxorubicin. As expected because of apoptosis induction, phosphorylation of c-MET decreases after doxorubicin treatment both in HGF pretreated and control cells. Moreover, in HGF pretreated cells phosphorylation is much lower, in accordance to the more relevant apoptosis (Fig. 5*C*).

We also investigated the persistence of c-MET/FAS complex after 24 h of doxorubicin treatment to determine if activation of FAS could affect complex stability. As shown in Fig. 5*D*, the c-MET/FAS complex remains stable after 24 h of doxorubicin treatment. This observation reinforces the proposed role of c-MET in increasing FAS efficiency.

We performed the same experiment also with 697 cell line, that does not express c-MET. As shown in Fig. 6*A*, and as expected because these cells do not express c-MET, 697 cells do not proliferate after stimulation with HGF (paired 2-tailed Student's *t* test, 24 h $p = 0.75$ and 48 h $p = 0.78$) (Fig. 6*A*, *upper panel*) and apoptosis rate, after 24 h of 0.5 μg/ml of doxorubicin treatment, does not change between HGF prestimulated and not prestimulated cells (Wilcoxon signed-rank test, $p = 0.84$) (Fig. 6*A*, *middle* and *lower panel*).

IL-3 Stimulation Does Not Increase Doxorubicin-induced Apoptosis—We promoted proliferation in REH cells also with IL-3 to demonstrate that the increased apoptosis observed after HGF stimulation and doxorubicin treatment is not a consequence of the increased cell proliferation because of HGF stimulation, but a specific effect of HGF activated c-MET on FAS.

First, we show that after 48 h IL-3-stimulated cells proliferate more than nonstimulated cells (paired 2-tailed Student's *t* test, $p = 0.01$) (Fig. 6*B*, *upper panel*). Then, as shown in Fig. 6*B*, *middle* and *lower panel*, we demonstrate that the percent of apoptosis does not differ between IL-3-stimulated and nonstimulated cells (Wilcoxon signed-rank test, $p = 0.054$). Of note in 6/10 experiments the percentage of apoptosis decreased in IL-3-stimulated cells (Fig. 6*B*, *middle panel*).

These results indicate that the increased cell proliferation after HGF stimulation in itself is not directly related to the higher apoptotic sensitivity observed after doxorubicin treatment, but this is specifically related to the effect of activated c-MET on FAS activity.

Activation of the FAS Pathway after Doxorubicin Treatment—Oligomerization of FAS following FAS-L engagement leads to the recruitment of FAS-associated proteins having death domains (*e.g.* FADD) and of the initiator procaspase-8 into a death-inducing signaling complex (DISC). Procaspase-8 then undergoes autoproteolysis to generate active caspase-8 (42).

As shown in Fig. 7*A*, we thus tested the activation of caspase-8 in HGF-pretreated and nonpretreated REH cells. After doxorubicin treatment, caspase-8 is cleaved and acti-

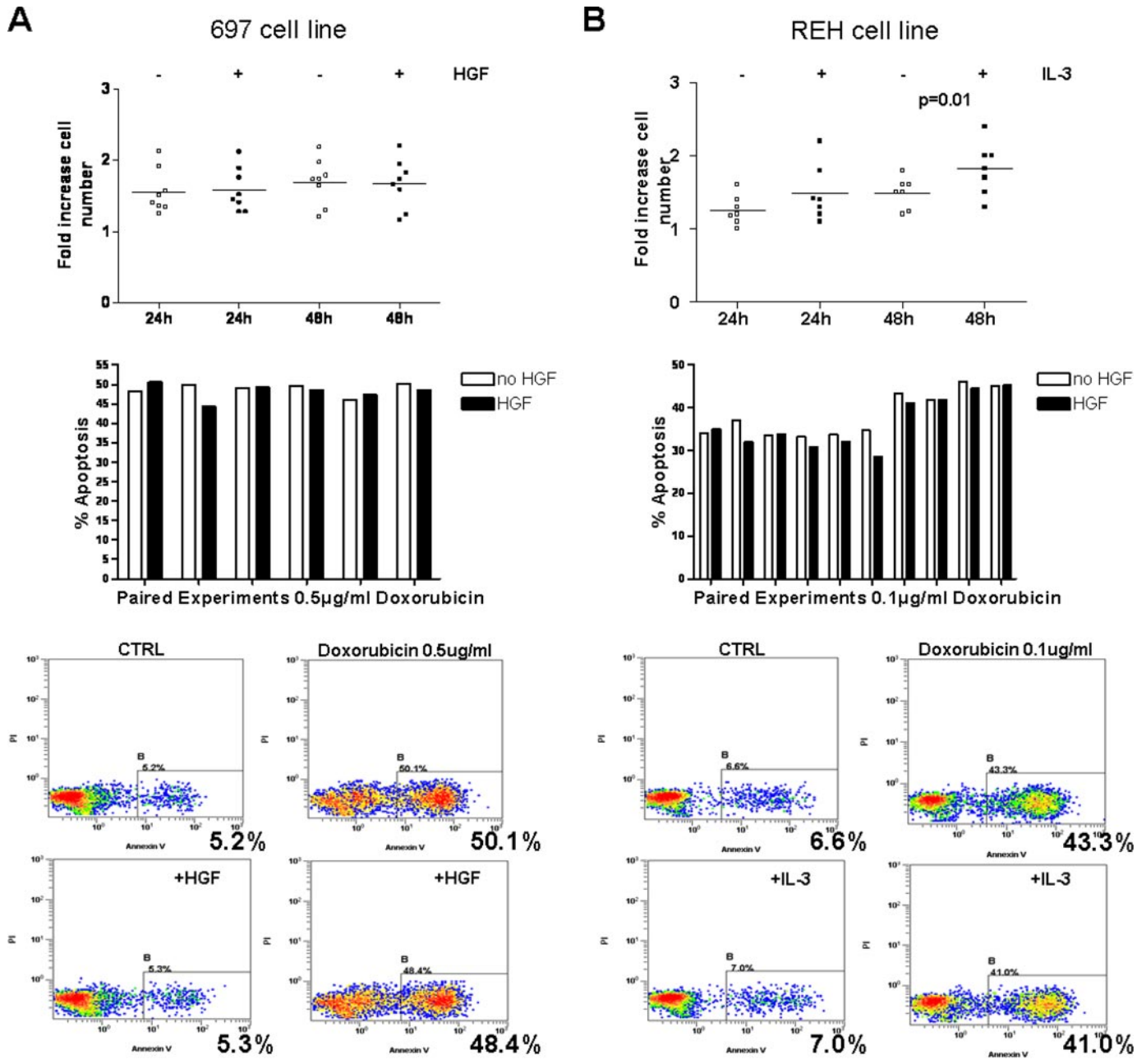

FIGURE 6. *A*, stimulation of 697 cells with HGF does not affect proliferation and apoptosis. *Upper panel*, after treatment with HGF, 697 cells proliferation does not increase (paired 2-tailed Student's *t* test, 24 h $p = 0.75$ and 48 h $p = 0.78$). Cells were starved for 18 h and subsequently treated with (■) or without (□) HGF (100 ng/ml) for 24 and 48 h. *Middle panel*, set of the six paired experiments at 0.5 μg/ml of doxorubicin is *highlighted* in the clustered column graph. *Lower panel*, example of apoptosis analysis measured by cytofluorimetric assay using Annexin V and PI staining. Where indicated, cells were exposed for 24 h to doxorubicin at 0.5 μg/ml and pretreated with HGF (100 ng/ml) for 48 h. Apoptotic cells are in the *lower part* of each plot, on the *right side* (Annexin V-positive and PI-negative). HGF treatment does not affect doxorubicin-induced apoptosis percentage (Wilcoxon signed-rank test, $p = 0.21$). *B*, increased proliferation of REH cells after IL-3 stimulation does not affect doxorubicin-induced apoptosis. *Upper panel*, increase of cell proliferation after treatment of REH cells with IL-3 (unpaired 2-tailed Student's *t* test, 48 h $p = 0.01$). Cells were starved for 18 h and subsequently treated with (■) or without (□) IL-3 (20 ng/ml) for 24 and 48 h. *Middle panel*, set of the 10 paired experiments at 0.1 μg/ml of doxorubicin is *highlighted* in the clustered column graph. *Lower panel*, example of apoptosis analysis measured by cytofluorimetric assay using Annexin V and PI staining. Where indicated, cells were exposed for 24 h to doxorubicin at 0.1 μg/ml and pretreated with IL-3 (20 ng/ml) for 48 h. IL-3 treatment does not affect doxorubicin-induced apoptosis percentage (Wilcoxon signed-rank test, $p = 0.054$).

vated both in pretreated and in control cells. However, caspase-8 activation is higher in HGF-pretreated cells after doxorubicin treatment, demonstrating that the FAS pathway is more active in these cells. Difference in the cleavage of caspase-8 is demonstrated also by densitometric analysis (Fig. 7*A*, *lower panel*). In Fig. 7, *B* and *C*, we report that there is no increase in caspase-8 activation in 697 cells (Fig. 7*B*) after stimulation with HGF and doxorubicin treatment, and in REH cells (Fig. 7*C*) after IL-3 stimulation and doxorubicin treatment. These results confirm that the FAS-mediated apoptosis pathway is more activated only when c-MET is HGF-stimulated. Thus, our data strongly suggest that c-MET activation sensitizes TEL-AML1 cancer cells to the FAS apoptotic pathway.

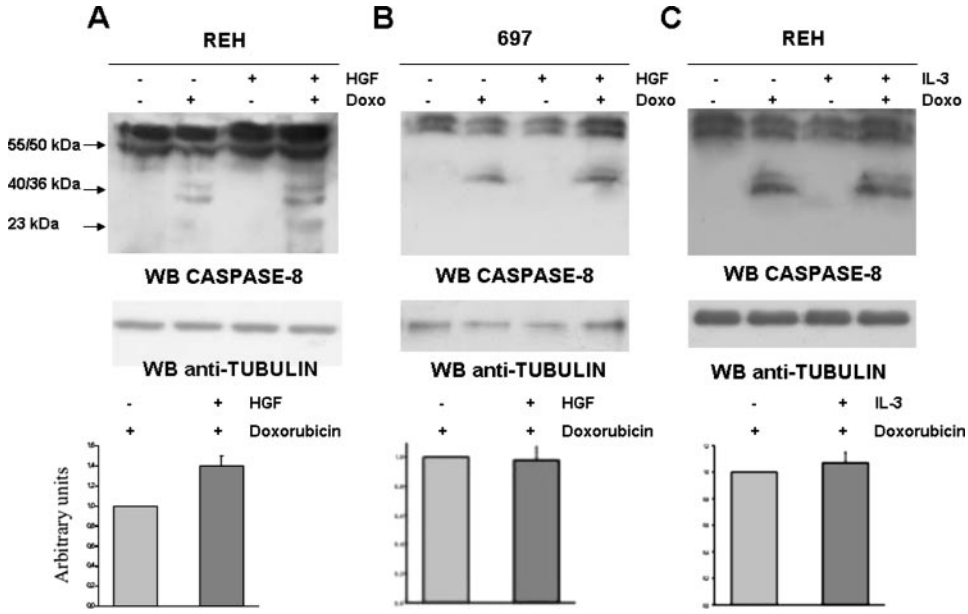

FIGURE 7. **Activation of caspase-8.** *A*, *upper panel*: activation of caspase-8 after doxorubicin treatment in the REH TEL-AML1 cell line. Cell lysates were analyzed by Western blot using a specific antibody that recognizes both procaspase-8 (55/50 kDa) and mature caspase-8 (40/36 kDa and 23 kDa). Cells were analyzed where indicated after HGF stimulation (100 ng/ml, 72 h) and doxorubicin treatment (0.1 μg/ml, 24 h). Membranes were stripped and reprobed with specific anti-α-tubulin antibody to control equal protein loading. *Lower panel*, densitometric analysis of the amount of cleaved caspase-8 in pretreated (+*HGF*, +*Doxorubicin*) and in control REH cells (−*HGF*, +*Doxorubicin*); data are the means ± S.E. *B*, *upper panel*: activation of caspase-8 after doxorubicin treatment in 697 cell line. Cells were analyzed where indicated after HGF stimulation (100 ng/ml, 72 h) and doxorubicin treatment (0.5 μg/ml, 24 h). *Lower panel*, densitometric analysis of the amount of cleaved caspase-8 in pretreated (+*HGF*, +*Doxorubicin*) and in control 697 cells (−*HGF*, +*Doxorubicin*). *C*, *upper panel*: activation of caspase-8 after doxorubicin treatment in REH cell line treated with IL-3. Cells were analyzed where indicated after IL-3 stimulation (20 ng/ml, 72 h) and doxorubicin treatment (0.1 μg/ml, 24 h). *Lower panel*, densitometric analysis of the amount of cleaved caspase-8 in pretreated (+*IL-3*, +*Doxorubicin*) and in control REH cells (−*IL-3*, +*Doxorubicin*).

DISCUSSION

Here we studied the expression of the c-*Met* gene in pediatric B-ALL, and we demonstrated that it interacts with FAS, thus sensitizing TEL-AML1 cells to doxorubicin treatment. Overexpression and mutations of this gene were reported previously in other hematological malignancies like acute myeloid leukemia (11) but no previous data exist concerning c-MET expression and function in any acute B lineage leukemia.

Here we report that c-*Met* is expressed in normal CD19+ B lymphocytes as well as in some, but not all, pediatric B-ALL patients. Interestingly, c-*Met* is predominantly expressed in t(12;21) TEL-AML1 leukemias. The t(12;21) (p13;q22) translocation occurs in ~25% of newly diagnosed childhood B-ALL patients, and it is the most common gene rearrangement yet identified in pediatric leukemias (27, 43–45). This leukemia subtype is primarily present in children 2–5 years of age and is thought to originate from preleukemic clones that very likely originate *in utero* and undergo a second transformation (46). Several studies (27–31) indicate that patients with TEL-AML1 have an excellent prognosis, ranging from 90 to 100% event-free survival rates. Expression of c-*Met* in all patient samples with TEL-AML1 translocation is remarkable because it is not present or present at lower levels in the other B-ALL specimens. The fact that TEL-AML1 cells respond to therapy and express c-MET at similar levels to normal B lymphocytes suggests that c-MET could play a physiological role in TEL-AML1 blast cells. It suggests also that the lack of c-MET expression in patients without TEL-AML1 could be related to the less favorable prognosis observed in these patients. Thus we were interested in understanding if c-MET could be involved in the successful response to chemotherapy of TEL-AML1 patients.

The life of a normal cell undergoes a cycle of proliferation, differentiation and ends with apoptosis. Physiological apoptosis can be induced by a multitude of stimuli including activation of death receptors such as FAS/CD95/APO-1. Defects in apoptosis have recently been explored as the most prominent molecular defect related to many cancers including leukemia. Cell lines resistant to therapeutic concentrations of cytotoxic drugs show marked down-regulation of FAS expression, suggesting that critical levels of FAS expression are required for apoptotic signaling and chemosensitivity (37). Along this line it has been shown that the death receptor FAS is underexpressed in leukemias (33, 34). In our samples we observed FAS underexpression with no difference between TEL-AML1 and non-TEL-AML1 patients. Our patients do not differ in terms of FAS expression, but they do in terms of c-MET expression. An influence on the apoptotic activity of FAS exerted by c-MET was observed in some epithelial cancer cells by the formation of a protein complex (25, 26). Our study investigated the presence of this complex in leukemia cells and whether variability in c-MET expression could influence FAS function. We observed, for the first time, that c-MET co-precipitates with FAS in normal B lymphocytes, in TEL-AML1 B-ALL patients and cell line.

To better understand the dynamic of this interaction, we first tried to physiologically stimulate c-MET with its ligand HGF. In doing this, we wanted to determine if the tyrosine kinase receptor could activate the downstream signal cascade while physically bound to FAS. In HGF-stimulated cells the phosphorylation of c-MET is increased, hence, FAS does not prevent HGF from binding its receptor. Stimulated cells increased their proliferation rate in an unequivocal manner, demonstrating that c-MET is able to activate the downstream signaling cascade in lymphocytic cells without any defects. Moreover the c-MET/FAS complex was not disrupted by HGF stimulation. These results indicate that c-MET activity is not influenced by FAS binding, and because the protein complex remains stable after HGF treatment, we questioned how c-MET activation could affect FAS-mediated apoptosis.

In leukemia (47) and neuroblastoma cells (48) it has been reported that the FAS pathway contributes to doxorubicin-

induced cell death, and we report that also in REH cell line doxorubicin involves FAS-mediated apoptosis (Fig. 4). Therefore we decided to stimulate apoptosis in REH cells using doxorubicin, to activate the FAS pathway and study c-MET influence on it. We show that HGF-prestimulated cells undergo apoptosis more than not prestimulated cells, thus demonstrating that a physiological activation of the c-MET pathway assists FAS in inducing apoptosis in TEL-AML1 leukemia cells. Interestingly, a high sensitivity to doxorubicin of TEL-AML1 tumor cells was previously observed by Frost *et al.*, in 2004 (45). We chose this approach since HGF activation of c-MET followed by treatment with doxorubicin is a physiological rather invasive way to study how these two proteins interact and influence cellular homeostasis. Additionally, growth stimulation with IL-3 does not result in increased apoptosis, demonstrating that increased cell proliferation alone does not influence apoptotic rate. Thus, the increased apoptotic percentage observed after combined HGF/doxorubicin treatment can be attributed to a stronger activation of the FAS pathway by stimulated c-MET. The stronger activation of caspase-8 after doxorubicin treatment only in HGF-pretreated REH cells supports our hypothesis that the increase of apoptosis observed in HGF-stimulated cells is dependent on stronger FAS activation by stimulated c-MET within the protein complex. Thus, it is activated c-MET that, interacting specifically with FAS, enhances apoptosis in doxorubicin-treated cells.

Our study is the first one that focuses on c-MET role in pediatric B acute lymphoblastic leukemia, and our results offer important and new insights of the HGF receptor c-MET role in these tumors. The observation that c-MET enhances FAS-mediated apoptosis in leukemia cells during drug therapy is an important new discovery in the role of the HGF receptor c-MET in pediatric leukemias, and provides new elements for understanding the mechanisms that regulate the favorable response to therapy observed in TEL-AML1 patients.

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Hepatocyte Growth Factor Receptor c-MET Is Associated with FAS and When Activated Enhances Drug-induced Apoptosis in Pediatric B Acute Lymphoblastic Leukemia with TEL-AML1 Translocation

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