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Establishment and phenotypic characterization of human U937 cells with inducible P210 BCR/ABL expression reveals upregulation of CEACAM1 (CD66a)

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Chronic myeloid leukemia (CML) is characterized by the expression of the P210 BCR/ABL fusion protein. The molecular mechanisms behind this oncogene-mediated hematological disease are, however, not fully understood. Here, we describe the establishment and phenotypic characterization of U937 cells in which P210 BCR/ABL can be conditionally expressed using tetracycline. The induction of BCR/ABL in the obtained clones resulted in a rapid phosphorylation of the STAT1, STAT3 and STAT5 molecules, consistent with the findings in other model systems. Phenotypic characterization of the clones revealed that BCR/ABL induces a slight decrease in the proliferation and viability, without a marked effect on cell cycle distribution, the rate of apoptosis or on cellular differentiation, as judged by several cell surface markers and capacity to reduce nitro blue tetrazolium. Interestingly, BCR/ABL was found to upregulate the expression of carcinoembryonic-related antigen (CEA)CAM1 (CD66a), which is a plasma membrane-linked glycoprotein belonging to the CEAs and involved in signal transduction and cellular adhesion. The expression of CEACAM1 was reversible upon imatinib treatment in BCR/ABL-expressing U937 cells as well as in BCR/ABL-positive K562 cells. The established cell lines may prove useful in further modeling and dissection of BCR/ABL-induced leukemogenesis.
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Introduction

The *BCR/ABL* oncogene originates from a reciprocal translocation, t(9;22)(q34;q11), which fuses the *BCR* gene on chromosome 22 with the *ABL* gene on chromosome 9. Depending on the location of the breakpoint in the *BCR* gene, either of the two fusion proteins P210 or P190 BCR/ABL is expressed. These proteins are mainly associated with chronic myeloid leukemia (CML) and acute lymphoid leukemia (ALL), respectively. Compared to the normal ABL tyrosine kinase protein both P210 and P190 BCR/ABL have an enhanced kinase activity, which is indispensable for the malignant transformation.[1–3]

CML is a disorder of the pluripotent hematopoietic stem cell and is characterized by an increase and premature release of primitive myeloid cells into the blood.[3,4] The expansion of the leukemic clone is thought to result from increased proliferation and/or from reduced apoptosis as well as from deregulated cell adhesion.[1–3] It has also been reported that BCR/ABL can induce differentiation without affecting proliferation,[5] and more recently retroviral transduction of BCR/ABL into primitive human hematopoietic progenitors suggest that BCR/ABL is capable of reprogramming the prior lineage commitment of these cells.[6]

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The exact mechanisms behind the BCR/ABL-induced signals for transformation are still largely unknown, but a number of signal transduction pathways such as the Ras, the PI-3 kinase and the JAK/STAT pathways have been shown to become activated by BCR/ABL.[2,7] The signal transducers and activators of transcription (STAT) molecules are involved in tyrosine kinase signaling. Upon phosphorylation they become activated, dimerize and translocate to the nucleus where they activate gene transcription.[8] BCR/ABL has been shown to mainly phosphorylate STAT5, but also STAT1 and STAT3.[9–11] In BCR/ABL-positive cells, these STAT molecules have been suggested to affect a number of different cellular functions, such as cell cycle progression and apoptotic activity.[12]

In the present study, we have generated human U937 cells expressing P210 BCR/ABL under the control of a tetracycline-inducible promoter. We report a detailed molecular and phenotypic characterization of these cells and upregulated cell surface expression of carcinoembryonic-related antigen (CEA)-CAM1 in response to the induction of BCR/ABL.

Materials and methods

Cell lines and culture conditions

The human monocytic cell line U937[13] and its subclones were cultured in RPMI 1640 with Glutamax-I (Invitrogen Corporation, Stockholm, Sweden) supplemented with 10% Tet system approved fetal calf serum (FCS; Clontech Laboratories, Inc., Palo Alto, CA, USA). *BCR/ABL*-transfected cell cultures were grown in the presence of 447 U/ml hygromycin B (Calbiochem, Calbiochem-Novabiochem Corporation, La Jolla, CA, USA) and 0.5 mg/ml geneticin (Roche Diagnostics, Mannheim, Germany). The human erythroleukemia cell line K562,[14] used as positive control for P210 BCR/ABL and STAT5 phosphorylation, was cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS and 2 mM L-glutamine (Invitrogen Corporation). Cells were grown in a humidified 5% CO_2 atmosphere at 37°C. Exponentially growing cells were used for all experiments.

Vector construct and transfection procedure

The Tet-On Gene Expression System (Clontech Laboratories, Inc.) was used according to the manufacturer's instructions. At first, the established U937/Tet-On/A38 clone, carrying the pTET-On regulator plasmid conferring resistance to geneticin, was transfected with pTRE-Luc. Owing to its low luciferase expression in the absence of doxycycline and a 20- to 60-fold induction of luciferase in the presence of doxycycline, the U937/Tet-On/A38 clone was used for transfection with *BCR/ABL*. pTRE-P210*BCR/ABL* was constructed by digesting AB1/pCDE (kindly provided by Drs J Groffen and N Heisterkamp, Childrens Hospital Los Angeles, Los Angeles, CA, USA) with

*Eco*RI. The cDNA fragment containing the entire cDNA for P210 BCR/ABL was isolated and ligated into *Eco*RI-digested pTRE (Clontech Laboratories, Inc.) containing a 5′ tetracycline (tet)-responsive promoter. Multiple restriction enzyme digestions identified clones with cDNA positioned in a correct orientation.

Before transfection, cells were resuspended to a concentration of 4×10^6 cells/ml. The pTRE-P210*BCR/ABL* (10 μg) and the pTK-Hyg plasmids (1 μg) were cotransfected by electroporation using the Bio-Rad gene-pulser (Bio-Rad, Melville, NY, USA) with electrical settings of 260 V and 960 μF. After 2 days, cells were seeded in 96-well plates at a concentration of 0.05×10^6 cells/ml in the presence of 447 U/ml hygromycin B (Calbiochem). Control clones, that is, mock transfectants, were obtained by transfection with pTK-Hyg and pTRE. Following the expansion of individual cell clones, 0.5 mg/ml geneticin (Roche Diagnostics) was added to the cultures. BCR/ABL expression was induced by adding 1–2 μg/ml doxycycline (Sigma Chemicals, St Louis, MO, USA) to the culture medium and the level of induction was analyzed by Western blot analysis. To exclude the effects induced by doxycycline independent of BCR/ABL, the control cells were identically treated with doxycycline in all experiments.

Western blot analysis and assessment of STAT phosphorylation

Protein lysates were prepared from control and transfected cells after incubation with or without doxycycline (1–2 μg/ml). As positive control for STAT phosphorylation, mock cells were incubated with 2000 U/ml IFNα (Introna; Schering-Plough, Stockholm, Sweden). To generate lysates, 5×10^6 cells were washed in phosphate-buffered saline (PBS) and resuspended in 25 μl PBS plus 4 μl protease inhibitor (complete protease inhibitor cocktail tablets; Roche, Bromma, Sweden) and 75 μl Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) containing 5% β-mercaptoethanol. All samples were sonicated for about 10 s and stored at -20°C until usage.

The lysates were boiled for 5 min before separation of 20–30 μl extract on 7.5 or 12% Tris-HCl SDS-PAGE (Bio-Rad Laboratories) in 1 × running buffer (10 × Tris/glycine/SDS buffer; Bio-Rad Laboratories). Blotting was performed in 1 × transfer buffer (premixed 10 × Tris/glycine buffer, Bio-Rad Laboratories) containing 20% methanol using a semidry blotter (Kem-En-Tec, Copenhagen, Denmark) and Hybond-P nitrocellulose membranes (Amersham Pharmacia Biotech, Inc., Uppsala, Sweden). Membranes were blocked at room temperature in 1 × TBS-T (20 mM Tris base pH 7.6, 137 mM NaCl, 3.8 mM HCl and 0.1% Tween) plus 5% nonfat dry milk. Incubation with primary antibody was performed overnight in 1 × TBS-T plus 0.5% dry milk at 4°C. Primary antibodies used were: c-abl/Ab-3 (α-ABL; Calbiochem), Kat4c (detects CEACAM1, CEACAM6, CEACAM8 and CEA; DAKO, Glostrup, Denmark), 4/3/17 (detects CEACAM1 and CEA; Genovac, Freiburg, Germany), α-p-STAT1 (Y701), α-p-STAT3 (Y704), α-p-STAT5A/B (Y694/Y699), α-p-STAT6 (Y641) (Upstate Biotechnology, Lake Placid, NY, USA) and α-actin (C-2; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After washing, membranes were incubated for 1 h with either horseradish peroxidase (HRP)-linked α-mouse or α-rabbit (Amersham Pharmacia Biotech) antibody in 1 × TBS-T plus 0.5% dry milk at room temperature. Protein detection was performed with the enhanced chemiluminescence detection kit ECL + Plus (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Membranes were stripped in 100 mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl pH 6.7 for 30 min at 50°C under constant agitation, washed 2 × 10 min in 1 × TBS-T at room temperature and stored wet wrapped at 4°C until next usage.

Assessment of proliferation

Untreated and doxycycline (1 μg/ml)-treated control and transfected cells were grown at an initial concentration of 0.3×10^6 cells/ml in a medium supplemented with 10% FCS, but were thereafter not feeded. To test the effect of imatinib mesylate (also designated STI571) treatment, 1 μg/ml doxycycline and 1 μM imatinib mesylate (kindly provided by Dr E Buchdunger, Novartis, Basel, Switzerland) were added to the medium of BCR/ABL-transfected cells at the same time. The total number of cells was determined by daily counting in a Bürker chamber and viability was determined by trypan blue exclusion.

Assessment of cell cycle distribution

Control cells and transfected cells were incubated with or without 1 μg/ml doxycycline for 24, 48, 72 and 96 h. As a positive control for cell cycle arrest, the uninduced cells were incubated with 0.1 mM all-*trans* retinoic acid (ATRA) (Sigma-Aldrich, Stockholm, Sweden) for the same time points. Cells were washed in PBS and 50 μg/ml propidium iodide (PI) was added together with nuclear isolation medium in order to stain the nuclear DNA content. The samples were kept at room temperature in the dark for 5 min and before flow cytometric DNA content analysis was performed, the cells were incubated for at least 15 min at 4°C. Up to 20 000 nuclei were analyzed per sample in an Ortho Cytofluorograph (Ortho Diagnostic Systems, Raritan, NJ, USA) and cell cycle distribution was determined by applying the MultiCycle software (Phoenix Flow Systems, San Diego, CA, USA) on the DNA histograms, which were corrected for contribution of nucleic debris and aggregates.

Assessment of apoptosis

The determination of apoptosis was performed with Annexin V and PI staining (Annexin V–FITC apoptosis detection kit 1; BD Biosciences, Stockholm, Sweden). Control cells and transfected cells were incubated with or without 1 μg/ml doxycycline for 48, 72, 96 and 120 h. Apoptotic control cells were prepared by incubating uninduced cells with 10 pM human TNFα (kindly supplied by Dr GR Adolf, Ernst Boehringer Institut, Vienna, Austria), added 24 h after initiation of the experiment. Cells were washed in PBS and resuspended at a concentration of 5×10^6 cells/ml in 1 × binding buffer (included in the kit). Of this suspension, 100 μl was incubated with 5 μl each of Annexin V–FITC and PI in the dark for 15 min at room temperature. In all, 10 000 cells were analyzed for the expression of Annexin V in parallel with PI staining.

Assessment of differentiation

Pretreatment with 1 μg/ml doxycycline was carried out 24 h before initiating the induction of differentiation. The cells (0.2×10^6 cells/ml) were either incubated in the culture medium alone or in medium supplemented with 0.1 μM vitamin D3 (1-α-25-dihydroxycholecalciferol; a generous gift from Roche, Basel, Switzerland) or 10 μM ATRA (Sigma Chemicals) for 4 days. At

harvest, cells were subjected to the nitro blue tetrazolium (NBT) test. Cells were resuspended at a concentration of $0.5–1.0 \times 10^6$ cells/ml in RPMI 1640 supplemented with 20% FCS (Tet system approved; Clontech Laboratories, Inc.), 0.75 mg/ml NBT (Sigma Chemicals) and 0.15 μg/ml phorbol-12-myristrate-13 acetate (Sigma Chemicals) and incubated for 25 min at 37°C. The reaction was stopped by incubation on ice. Cytospin slides were prepared, stained with May–Grünwald–Giemsa and the percentage of cells able to reduce NBT, as seen by intracellular formazan deposits, was determined by counting at least 200 cells in light microscopy.

Assessment of cell surface marker expression

Following incubation in the absence or presence of doxycycline (1 μg/ml), control cells and transfected cells were washed in PBS and resuspended to 5×10^6 cells/ml. Of this suspension, 50 μl was incubated with 5 μl of the following antibodies: control IgG1-FITC/IgG1-PE, HLA DR-FITC, CD11c-PE, CD16-PE, CD34-PE, CD49f-FITC, CD49d-PE, CD56-PE, CD117-PE (all from BD Biosciences), CD11a-FITC, CD11b-PE, CD13-PE, CD14-PE, CD15-FITC, CD24-FITC, CD33-PE, CD45-FITC, CD62L-FITC, CD66abce-FITC (Kat4c; detects CEACAM1, CEACAM6, CEACAM8 and CEA) (all from DAKO) and CD44-FITC, CD54-FITC (from Immunotech, Marseille, France), for 10 min at room temperature under constant agitation. The cells were washed and fixed in 1% paraformaldehyde before flow cytometric analysis (FACScan; Becton Dickinson) was performed. For each pair of antibodies, 10 000 cells were analyzed.

Semiquantitative RT-PCR analysis and sequencing

cDNA was synthesized using 5 μg Trizol-extracted (Invitrogen Corporation) total RNA, random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen Corporation). Gene sequences for the CEAs were retrieved by the CEA family homepage at http://cea.klinikum.uni-muenchen.de/ and primers were designed using the Oligo program (National Bioscience, Inc., Plymouth, MN, USA) (Table 1). The *CEACAM1* gene has many different splicing variants and thus primers were designed to recognize two of the most common, the 3L and the 4L variants. PCR amplifications were performed in 50 μl reaction mixtures consisting of 1 × PCR buffer (Invitrogen Corporation), 1.25 mM $MgCl_2$, 0.8 mM dNTPs, 1 U Platina *Taq* polymerase (Invitrogen Corporation), 0.5 mM of the forward and reverse primers (Invitrogen Corporation) and 1–2 μl cDNA template of a two-fold dilution series (1:1; 1:2; 1:4; 1:8). The reactions were run in a thermal cycler (PTC-200, MJ Research Inc., MS, USA) for 20–30 cycles and the product (15 μl) was analyzed on a 1.5% agarose gel containing 0.2 μl/ml EtBr.

As positive control for CEACAM1 and CEA transcription, cDNA from mock cells grown for 3 days in medium supplemented with 10 μM ATRA (Sigma-Aldrich) and from epithelial HaCaT cells (kindly provided by Dr NE Fusenig, German Cancer Research Center, Heidelberg, Germany), respectively, were used.[15] To test the effect of imatinib mesylate (also designated STI571) on CEACAM1 transcription, 1 μg/ml doxycycline and 1 μM imatinib mesylate were added to the medium of BCR/ABL-transfected cells at the same time. The cells were then cultured for 24 h before RNA extraction. K562 cells were cultured with or without 1 μM imatinib mesylate and RNA was extracted after 5, 10, 24, 48 and 72 h, respectively, followed by RT-PCR analysis.

For sequencing, the 3L and 4L CEACAM1 PCR products were excised from the agarose gel, purified using the QIAquick Gel Extraction kit (Qiagen, VWR International AB, Stockholm, Sweden) and quantified using lambda DNA. The amplification was performed in 20 μl reactions containing BigDye mix (Applied Biosystems, Warrington, UK), 5–7 μl template and 3.2 μM of the forward CEACAM1 primer at 55°C for 26 cycles. The samples were purified using the QIAquick nucleotide removal kit (Qiagen), the DNA was precipitated and sequencing was performed in an ABI PRISM 3100 DNA sequencer (Applied Biosystems) as described by the manufacturer.

Results

Establishment of U937 clones with inducible BCR/ABL expression

Two clones, designated U937-P210*BCR-ABL*/c6 and e9, were established from two independent transfections. Both clones expressed P210 BCR/ABL upon doxycycline induction, but the

Table 1 Primers used for quantitative RT-PCR analysis

Transcript	*GenBank accession no.*	*Direction*	*Primer sequence (5′–3′)*	*Primer position*	*Fragment length (bp)*
CEACAM1-3L	X14831	F	GCA ACA GGA CCA CAG TCA AGA	995–1015	238
		R	CAC TCA GGA CCA CTC CAA TGA	1212–1232	
CEACAM1-4L	NM_001712	F	GCA ACA GGA CCA CAG TCA AGA	995–1015	526
		R	CAC TCA GGA CCA CTC CAA TGA	1500–1520	
CEACAM6	NM_002483	F	CAG GAA GAC TGG CAG ATT GG	1101–1120	441
		R	CTT GTC AAT CCC AAC GTT TTA CA	1519–1541	
CEACAM8	NM_001816	F	CGC AAC AGG ACC ACA GTC AG	992–1011	882
		R	TCC CTA CCT GAC TGC CAC AGA	1853–1873	
CEA	NM_004363	F	TAT ACG TGC CAA GCC CAT AAC	1003–1023	937
		R	ACT CGT CTT ACC TTT CGG GAG	1919–1939	
β-actin	NM_001101	F	CCT CGC CTT TGC CGA TCC	25–42	626
		R	GAC TGA CTA CCT CAT GAA GAT CC	628–650	

F, forward; R, reverse.

c6 clone showed a more distinct expression (Figure 1). No BCR/ABL protein was detected in uninduced c6 cells, whereas e9 cells showed weak leakage as determined by Western blot analysis (data not shown). The P210 BCR/ABL protein was already detected 6 h after doxycycline induction of the c6 cells and the expression typically reached its maximum at 24 h. In the mock clones, used as controls to exclude a doxycycline effect, no BCR/ABL protein was detected.

STAT pathway is activated by the P210 BCR/ABL protein

As the BCR/ABL protein has been shown to activate constitutively some of the STAT molecules through tyrosine phosphorylation,[9–11] we investigated if these transcription factors were also phosphorylated in the established c6 and e9 clones. At the uninduced state no STAT activation could be detected in the *BCR/ABL*-transfected clones. However, following BCR/ABL expression a clear phosphorylation of STAT1, STAT3 and STAT5 was detected in both clones (Figure 2 and data not shown). No STAT6 activation was found either in BCR/ABL positive cells or in control cells (data not shown).

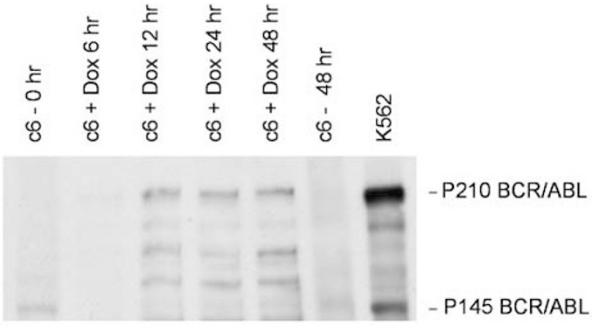

Figure 1 Kinetics of P210 BCR/ABL expression following the doxycycline induction of the c6 clone. At different time points after the addition of 1 μg/ml doxycycline, aliquots of cells were lysed and subjected to Western blot analysis using an ABL antibody. The P145 ABL and the P210 BCR/ABL proteins are indicated. K562 cells, expressing endogenous BCR/ABL, were used as positive control for P210 BCR/ABL.

Expression of BCR/ABL induces a slight decrease of proliferation and viability

BCR/ABL is thought to contribute to the expansion of the leukemic clone through enhanced proliferation and/or prolonged viability.[2] Therefore, we determined the growth rate and viability of the transfected cells maintained either in the presence or absence of doxycycline. Following BCR/ABL induction, both the c6 and the e9 clones showed a slightly decreased proliferation rate, accompanied by a slightly lower viability of the c6 clone (Figure 3). Mock cells treated with doxycycline also responded with decreased proliferation, although not to the same extent (Figure 3). Despite the fact that it seems unlikely that imatinib mesylate, at least with the used concentration, would be capable of fully blocking the activity of BCR/ABL following doxycycline induction, we also tested whether the effects of BCR/ABL on proliferation and viability were reversible upon treatment with this compound. Although a slight effect on these parameters was noted, a significant reversal was difficult to prove given the relatively small differences between BCR/ABL-induced effects and the growth-inhibiting effects on U937 cells caused by doxycycline and imatinib treatment only (data not shown).

Expression of BCR/ABL does not affect cell cycle distribution or apoptosis

To investigate if the slightly reduced proliferation rate found due to BCR/ABL expression could be the result of G1/G0 arrest or increased apoptosis, we performed cell cycle distribution analysis and Annexin V staining. Following BCR/ABL induction, none of the two transfected clones arrested in G1/G0 or showed any other marked alterations in cell cycle distribution, nor could any change in the rate of apoptosis be detected (data not shown). After incubation with ATRA, a well-known inducer of differentiation in U937 cells,[16,17] virtually all *BCR/ABL*-transfected cells as well as the mock control cells were arrested in the G1/G0 phase (data not shown). Following incubation with TNFα,

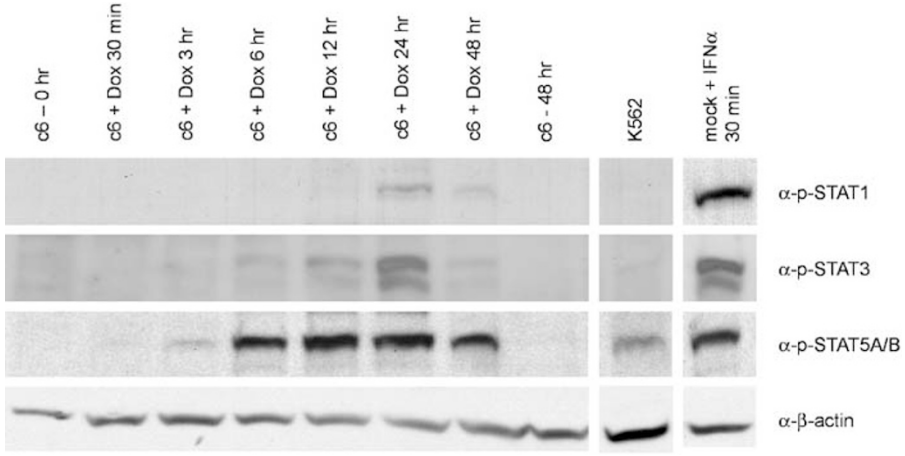

Figure 2 Western blots showing STAT phosphorylation. STAT1, STAT3 and STAT5 phosphorylation in the BCR/ABL-transfected c6 clone at different time points after induction are shown. K562 cells, as well as IFNα-treated mock cells, were used as positive controls for STAT5 phosphorylation. The latter control also showed STAT1 and STAT3 activation. Equal loading was verified using an actin antibody.

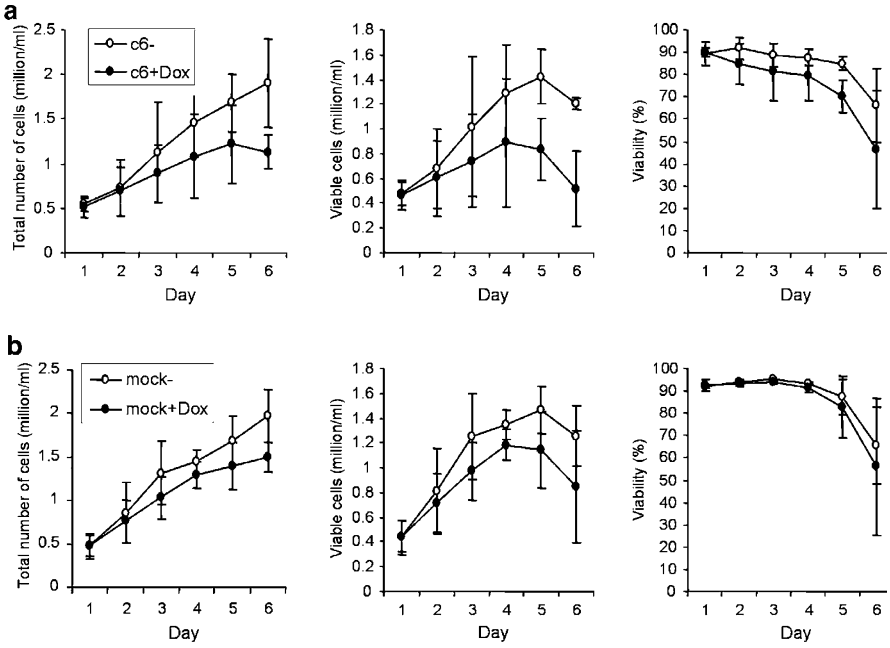

Figure 3 Proliferation and viability assessed by trypan blue exclusion. BCR/ABL was induced by adding 1 μg/ml doxycycline to the culture medium and cells were grown for 6 days, at an initial concentration of 0.3×10^6 cells/ml, without adding fresh medium. (a) The induction of BCR/ABL (c6 + dox) resulted in a decreased proliferation, as well as in a slightly lower viability for the c6 clone. (b) Mock cells treated with doxycycline also responded with decreased proliferation, although not to the same extent. Mean values from at least three separate experiments are shown. Bars indicate standard error of the mean (s.e.m.).

a well-known inducer of apoptosis, the Annexin V assay detected a high degree of apoptotic cells, verifying the reliability of the assay to detect apoptotic cells. The mock cells showed no marked difference between untreated and doxycycline-treated cells in either experiment (data not shown).

Effect of BCR/ABL expression on cellular differentiation of U937 cells

To study the capacity of BCR/ABL to interfere with differentiation, we used the NBT reduction test, which detects late stages of differentiation. At first, we determined if BCR/ABL could stimulate the differentiation of U937 cells, as previously described in stable M1-transfected cells,[5] but the NBT reducing capacity was found to be less than 1% (Table 2). To study if BCR/ABL potentially could interfere with vitamin D3 or ATRA-induced differentiation, c6 cells were cultured in the presence of these compounds. A varying degree of NBT reducing activity was observed between the *BCR/ABL* transfectants and the control cells, but no marked difference could be detected between untreated and doxycycline-treated c6 and mock cells, respectively (Table 2).

Table 2 NBT reduction test of U937 cells expressing BCR/ABL

	% NBT reducing cells			
Cultured in medium plus	*c6−*	*c6+dox*	*mock−*	*mock+dox*
—	<1	<1	<1	<1
ATRA	3.9	2.4	19.7	12.8
Vit D3	16.8	12.7	90.7	89.6

Cells were incubated with 10 μM ATRA and 0.1 μM vit D3 for 4 days, respectively, before determining NBT reducing capacity. ATRA, retinoic acid; dox, doxycycline; NBT, nitro blue tetrazolium; vit D3, vitamin D3.

BCR/ABL induces the expression of CEACAM1, which is reversible upon imatinib mesylate treatment

While the reduction of the NBT compound is a reliable but late marker of differentiation, studies of cell surface antigens are rather reflecting the phenotype of different cell stages along the differentiation pathway. To investigate if BCR/ABL is able to induce a differentiation response not detectable by the NBT reduction test, we studied the expression of a number of myeloid cell surface antigens using FACS analysis. The only alteration identified was an increased expression of CEAs, as detected by the Kat4c antibody (recognizing CEACAM1, CEACAM6, CEACAM8 and CEA). For the c6 clone, the greatest increase was detected 48 h after induction. At this time point, the percentage of cells positive for CEA raised from 1% at the uninduced state to around 13% after the induction of BCR/ABL (Figure 4). A similar trend, although not that marked, was also seen in e9 cells (data not shown).

As the Kat4c antibody recognizes different subgroups of the CEAs, Western blots were performed to determine which member of this family was responsible for the upregulation. At first, the same antibody (Kat4c) as used in the FACS analysis was used in Western blot experiments to confirm the deregulated expression. A band of approximately 160 kDa was clearly visible at 24 h after BCR/ABL induction. Using specific antibodies, this band was shown to correspond to either CEACAM1 or CEA (both detected with the 4/3/17 antibody; Figure 5), while no expression of CEACAM6 or CEACAM8 could

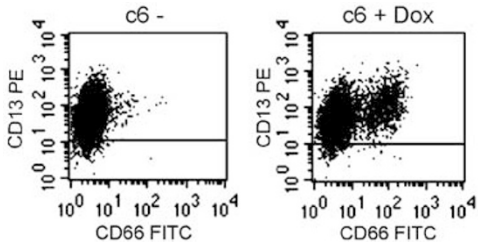

Figure 4 FACS analysis of CD13 and CD66 expression on uninduced (c6−) and BCR/ABL-positive (c6 + dox) c6 cells. Whereas CD13 expression was unchanged, the expression of CD66 (the CD66/Kat4c antibody recognizes CEACAM1, CEACAM6, CEACAM8 and CEA) was increased upon BCR/ABL expression as seen by the shift of the cell population to the right. The greatest increase was detected 48 h after BCR/ABL induction, when the CD66 expression rose from 1% for uninduced cells to around 13% for BCR/ABL-expressing cells. One representative experiment out of three from the 48 h BCR/ABL induction is shown.

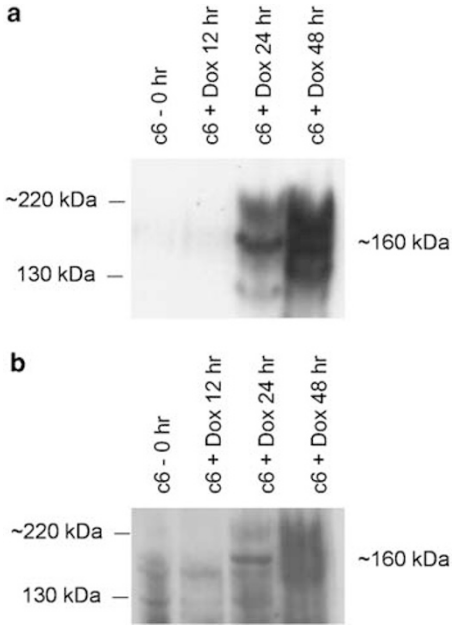

Figure 5 Western blots showing increased expression of the CEAs. (a) The broad Kat4c antibody was used to show upregulation following BCR/ABL induction with 1 μg/ml doxycycline of the c6 clone. Among several bands, a more distinct band of approximately 160 kDa was detected. (b) Using the 4/3/17 antibody, this band was shown to correspond to either CEACAM1 or CEA. The other bands most likely represent different isoforms or a different glycosylation pattern.

be detected (data not shown). Bands of other molecular weights were also detected, most likely representing splicing variants or differences in glycosylation pattern.

Semiquantitative RT-PCR and sequencing confirmed transcriptional upregulation of CEACAM1 (Figure 6). A slight increase of CEACAM6 and CEA transcripts was also detected, while no CEACAM8 transcript could be seen (data not shown). As seen in Figure 6, CEACAM1 transcription was induced following the BCR/ABL induction of c6 cells. In e9 cells, a slight CEACAM1 expression was also seen in the uninduced samples, probably resulting from a leakage of the BCR/ABL protein in this clone. Following treatment with the BCR/ABL inhibitor imatinib

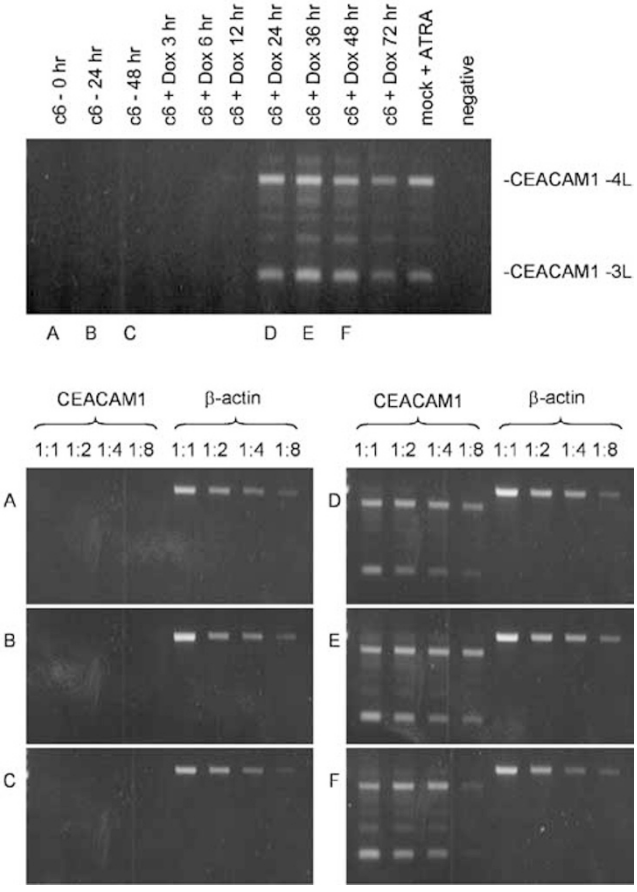

Figure 6 Semiquantitative RT-PCR analysis showing upregulated CEACAM1 transcription as a result of BCR/ABL expression. CEACAM1 primers were designed to detect two of the most common splicing variants, CEACAM1-3L and CEACAM1-4L. The top of the figure shows CEACAM1 expression in different samples of c6 cells, as well as in the positive control cells (mock + ATRA). At the bottom, serial dilutions of the template cDNAs (1:1, 1:2, 1:4, 1:8), followed by PCR analysis of CEACAM1 and β-actin, are depicted for the A–F samples indicated at the top of the figure, showing that the method allows a semiquantitative estimation of the expression levels of CEACAM1.

mesylate, a clear decrease of the CEACAM1 expression was seen indicating that the kinase activity of BCR/ABL is necessary for the induction of CEACAM1 (Figure 7).

CEACAM1 expression in K562 cells is reversed by treatment with imatinib mesylate

To study if the effect of BCR/ABL on CEACAM1 expression in c6 and e9 cells was extendable to other cells expressing the same fusion gene, the expression of CEACAM1 in K562 cells, following imatinib treatment, was investigated by semiquantitative RT-PCR analysis. A clear decrease in CEACAM1 expression was detected, indicating that BCR/ABL also regulates CEACAM1 expression in another cellular context (Figure 8). Serial dilutions of the cDNA used for RT-PCR analysis showed that the assay allowed a semiquantitative estimation of the expression levels of CEACAM1 (data not shown).

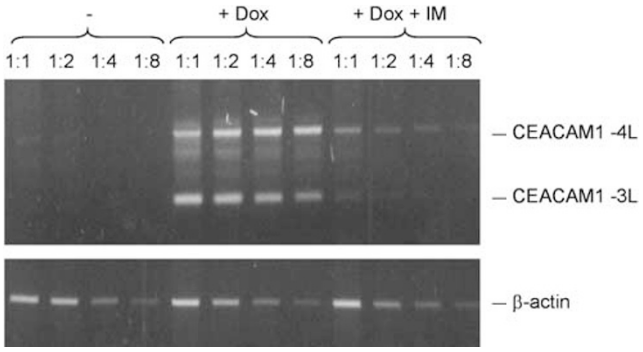

Figure 7 RT-PCR analysis of CEACAM1 expression following imatinib mesylate treatment. CEACAM1 transcription was shown to be reduced following culturing of BCR/ABL-positive c6 cells in 1 μM imatinib mesylate for 24 h. Treatment with imatinib mesylate was initiated at the same time as BCR/ABL was induced with 1 μg/ml doxycycline. As shown by serial dilutions of the template cDNAs (1:1, 1:2, 1:4, 1:8), the PCR conditions used allow a semiquantitative estimation of the CEACAM1 expression levels.

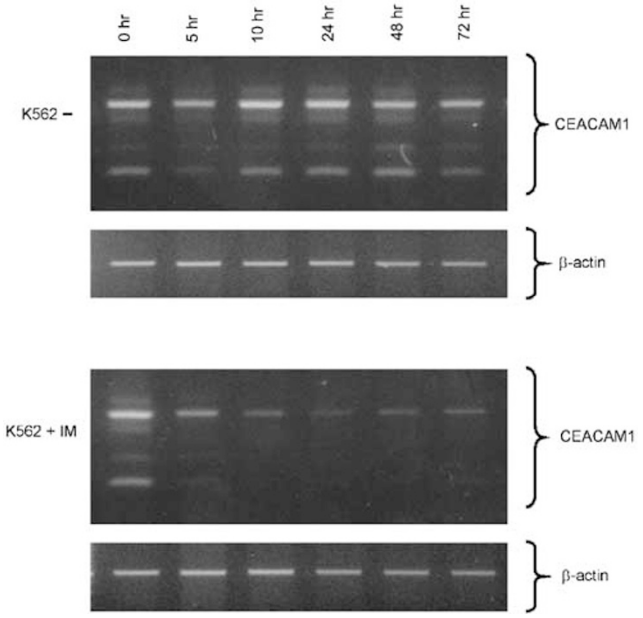

Figure 8 RT-PCR analysis showing reversible CEACAM1 expression in K562 cells treated with imatinib mesylate. K562 cells cultured in 1 μM imatinib mesylate containing medium for different time points show a clear decrease of CEACAM1 expression compared to untreated cells. The decrease is apparent already 5 h after the addition of the imatinib drug. The amplification of β-actin serves as a control for equal amounts of cDNA.

Discussion

The transforming potential of P210 BCR/ABL is likely to be mediated by the activation of a number of signal transduction pathways and over recent years many different experimental systems have been developed to study how BCR/ABL elicits its transforming activity. Model systems used include, for example, established Ph-positive cell lines, *BCR/ABL*-transfected murine and human hematopoietic cell lines, retroviral and transgenic animal models as well as primary CML cells.[2,18–21] Most *BCR/ABL*-transfected cell lines described to date, however, show a stable expression of BCR/ABL and only a few cell lines of murine origin (32D, Ba/F3 and FDCPmix) have been described in which this fusion gene can be conditionally expressed.[22–26] Conditional expression offers a potential advantage in that early effects of the introduced gene can be monitored in a rather controlled manner, and the risk of selecting for secondary mutations of critical genes/pathways given a possible repressive or growth-inhibiting effect of the transfected gene is likely to be lower.

To establish cells with inducible BCR/ABL expression, we have chosen the human monocytic cell line U937, which can be induced to differentiate into cells of monoblast- or macrophage-like character using various compounds.[13,16,17] Two clones, designated c6 and e9, were obtained, the c6 clone showing a tight regulation of BCR/ABL, whereas the e9 clone showed a faint leakage. The c6 clone also yielded a higher level of BCR/ABL expression upon doxycycline induction than the e9 clone. The expression of BCR/ABL resulted in a rapid phosphorylation of STAT1, STAT3 and STAT5, but not of STAT6, in both clones. These results are in agreement with previous studies, showing that STAT5, and to a lesser extent STAT1 and STAT3, are constitutively activated in most P210 BCR/ABL-positive cell lines and primary CML cells, whereas STAT6 only is activated by P190 BCR/ABL.[9–11,27] The STAT proteins are believed to regulate normally cell growth, differentiation and apoptosis and their deregulation by BCR/ABL, in particular of STAT5, is believed to be an important pathway by which this chimeric oncogene mediates its transforming activity.[28–32]

The initial chronic phase (CP) of CML is characterized by a vast expansion of myeloid cells at all stages of differentiation.[1,3] This accumulation is likely to be caused by an imbalance between signals linked to cellular proliferation and/or apoptosis.[2,18] In general, however, the proliferative effect of BCR/ABL is limited and cell kinetic studies *in vivo* do not indicate that CML cells proliferate more rapidly than their normal counterparts.[33,34] The antiapoptotic effect elicited by BCR/ABL in cell lines[35,36] is controversial since it has not been possible to recapitulate this effect in primary CML cells.[37,38] Upon induction of BCR/ABL in U937 cells, we observed a slight decrease in the cellular proliferation rate. To investigate if the slight proliferative decrease could be the result of G1/G0 arrest or an increased rate of apoptosis, we performed cell cycle distribution analysis and Annexin V staining, but no marked effects were noted. Given the limited sensitivity of these assays, subtle effects on these parameters can, however, not be ruled out.

We also studied whether BCR/ABL could promote cellular differentiation or interfere with vitamin D3- or ATRA-induced differentiation of U937 cells. Many previous functional studies were performed in murine cells (eg Ba/F3 cells), which have lost their ability to differentiate, and a possible effect of BCR/ABL on cellular differentiation could not have been detected in such cells.[22,24,39,40] The incubation of U937 cells with either vitamin D3 or retinoic acid induces terminal differentiation, growth inhibition and expression of cell surface markers, for example, CD14 and CD11c, associated with monocytic differentiation.[16,17,41] Using the NBT reduction test, a method detecting relative late stages of differentiation, and FACS analysis of a number of surface antigens, we failed to detect any clearcut alterations upon BCR/ABL induction alone, nor did we observe any interference or enhancement by BCR/ABL of vitamin D3- or ATRA-induced differentiation. The latter results are, however, most likely influenced by the observation of a diminished vitamin D3- and ATRA-inducibility, not related to BCR/ABL, obtained upon establishment of the c6 line. Indeed, a facilitating

effect by BCR/ABL on cellular differentiation has been described in murine myeloid M1 leukemia cells[5] and in human MO7 cells.[42]

Interestingly, BCR/ABL expression induced a marked upregulation of CEACAM1 in *BCR/ABL*-transfected U937 cells and the effect was shown to be reversible following treatment with imatinib mesylate, a relatively specific inhibitor of BCR/ABL.[43,44] Reversible CEACAM1 expression was also found in the BCR/ABL-positive K562 cell line, supporting the indication of either a direct or indirect effect of BCR/ABL on CEACAM1 regulation. CEACAM1 is a cell surface antigen belonging to the family of CEAs, which is a subgroup within the immunoglobulin superfamily. The CEA family comprises a large number of genes including the CEACAM1 (CD66a), CEACAM6 (CD66c), CEACAM8 (CD66b) and CEA (CD66e) subgroups.[45–47] The normal tissue distribution differs for these four molecules, whereas CEACAM1 and CEACAM6 show a broad expression pattern, CEACAM8 and CEA are expressed primarily on granulocytes and epithelial cells, respectively. Alterations in the expression pattern for CEA family members have been reported in various tumors, such as colon and breast carcinomas.[45,47–49]

The *CEACAM1* gene is the most well-conserved member of the CEA family and encodes a transmembrane cell adhesion molecule expressed in epithelia, vessel endothelia, granulocytes, macrophages and in T and B cells. Alternative splicing generates several isoforms where the two most common, the CEACAM1-L and CEACAM1-S, differ in the length of their cytoplasmic domain. Of these isoforms, human leukocytes only express the L variant with either three (CEACAM1-3L) or four (CEACAM1-4L) IgG loops, respectively.[45–47,50] It has been suggested that the tyrosine kinase activity associated with CEACAM1 may play a role in signal transduction and cellular adhesion, and that CEACAM1 might affect many important regulatory functions, such as proliferation, apoptosis, differentiation, angiogenesis, immune responses, granulocyte activation and polarization of epithelial cells.[45,47,51–55] Moreover, CEACAM1, like BCR/ABL, has been shown to associate with the actin cytoskeleton, possible by interacting with paxillin.[56–60]

In the hematopoietic system no detailed studies on the expression of CEACAM1 in different cellular compartments are available, but during normal hematopoiesis the expression pattern of the CEACAM1, CEACAM6 and CEACAM8 molecules has been shown to change with different maturation stages, first appearing at the promyelocytic stage and then showing increased expression on myelocytes and metamyelocytes.[61,62] An interesting observation in this context is that CEAs have been shown to be aberrantly expressed in ALL, preferentially in Philadelphia (Ph)-positive cases.[63] Furthermore, a monoclonal antibody (KOR-SA3544), later identified as CEACAM6,[64] was shown to react with all 26 investigated Ph-positive ALL cases, but only with a minority of Ph-negative ALL and AML.[65] Needless to say, however, further studies are needed before a firm conclusion can be made on mechanisms behind the observed upregulation of CEACAM1 by BCR/ABL. Hence, it is well possible that BCR/ABL signaling results in a direct upregulation of CEACAM1 or that a slight shift in the differentiation of U937 cells is obtained, resulting in an indirect upregulation of this molecule. The latter assumption is supported by studies showing that CEACAM1 is upregulated following ATRA-induced differentiation of U937 cells.[15]

During recent years, it has become increasingly clear that the cellular context, in which BCR/ABL is expressed, has significant impact on how cellular behavior is altered. Hence, extrapolation from studies in hematopoietic cell lines to primary CML cells must be made with caution. Nevertheless, the establishment of U937 cells with conditional expression of BCR/ABL may prove useful in the further elucidation of BCR/ABL-mediated signaling, in particular, of early effects elicited by this fusion gene.

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