CRIM1 is Expressed at Higher Levels in Drug-resistant than in Drug-sensitive Myeloid Leukemia HL60 Cells

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Abstract. *Aim: The aim of this study was to explore possible differences in the mRNA expression levels of CRIM1, SMAD5, BMP4 and BMP7 in sensitive (S) and multidrug-resistant (R0.5) myeloid leukemia HL60 cells. Materials and Methods: HL60S and HL60R0.5 cells were exposed to daunorubicin (DNR) or cytarabine (Ara-C). Results: Baseline levels of CRIM1 were found to be 15-fold higher in HL60R0.5 than in HL60S. Sixteen hours of exposure to DNR resulted in a 5.6-fold increase in CRIM1 levels in HL60S. Exposure to either DNR or Ara-C resulted in modest increases in CRIM1 levels in HL60R0.5. Similarly, baseline levels of SMAD5 and BMP4 were higher in HL60R0.5 than in HL60S cells. Analysis of the drug SMAD5-resistance marker permeability-glycoprotein (Pgp) revealed that CRIM1 and Pgp exhibit a covariance pattern of expression. Conclusion: This study demonstrated that CRIM1 is expressed at high levels in resistant leukemia cells, indicating that CRIM1 may play a role in drug-resistance.*

Several mechanisms involved in resistance to cytostatic drugs have been identified in myeloid leukemia cells. The transmembrane protein permeability-glycoprotein (Pgp), a member of the ATP-binding cassette (ABC) family, plays a key role in one of the most extensively studied mechanisms. In acute myeloid leukemia (AML), overexpression of Pgp has been shown to confer resistance to a large number of unrelated compounds, which is known as multidrug resistance, and has been demonstrated to be a predictor of poor prognosis (1, 2). Intrinsic or acquired multidrug resistance, due to overexpression of Pgp and other mechanisms, is the major cause of treatment failure in AML and the development of treatments that overcome this resistance has been the aim of decades of research.

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The cysteine rich transmembrane bone morphogenetic protein regulator 1 (chordin-like) (CRIM1) is a cell-surface transmembrane protein with a large extracellular moiety containing six von Willebrand-like, cysteine-rich repeats and an insulin-like growth factor-binding protein motif (3, 4). In human, the highest levels of *CRIM1* mRNA have been detected in placenta and kidney (3). The biological significance of CRIM1 during development of, for example, the eye, the central nervous system and the kidneys, has been firmly established (5-7).

In structure, CRIM1 resembles other developmentally important proteins (such as uterine sensitization associated gene-1) that are known to interact with bone morphogenetic proteins (BMP) (8). BMPs signal through the SMAD pathways to regulate the fate of hematopoietic progenitor cells and stem cells (9). CRIM1 has been shown to interact with, among others, BMP4 and BMP7, by tethering the inactive pre-forms of BMP to the extracellular face of the plasma membrane in COS-1 kidney cells. CRIM1 has been reported to have at least three effects on BMPs in COS-1 kidney cells: (i) CRIM1 decreases the production and processing of pre-forms of BMPs into mature BMPs, (ii) CRIM1 reduces secretion of mature BMPs, and (iii) CRIM1 is associated with a proportion of secreted BMPs, either as a complex or tethered to the cell surface (4). Whether CRIM1 acts by similar modes of action in myeloid hematopoietic cells as in COS-1 kidney cells is unknown and remains to be elucidated.

In a microarray study, Ichikawa *et al*. showed that the expression of CRIM1 mRNA varied in leukemia cells from patients diagnosed with different forms of AML (10). For instance higher levels of CRIM1 were expressed in cells with the mutation inv (16) than in cells with the mutation t(8;21), both of which are cytogenetic aberrations associated with relatively good prognosis in AML (10). These results suggest that the expression of CRIM1 might vary in leukemia cells with different genotypes and resistances to chemotherapy. The expression of CRIM1 in leukemia cells with different drug-resistant phenotypes has not been studied. The aim of the present study, therefore, was to investigate whether there

are differences in the mRNA expression levels of CRIM1, and the mRNA levels of the related proteins SMAD5, BMP4 and BMP7, between leukemia cells that are sensitive to cytostatic drugs and leukemia cells that are multidrug resistant due to high expression levels of Pgp.

Materials and Methods

Cell lines and culture conditions. Human leukemia HL60 cells that are highly sensitive to doxorubicin (HL60 S), and the sub-cell line HL60 R0.5, which are resistant to 0.5 μM doxorubicin (11), were grown in suspension in Roswell Park Memorial Institute (RPMI) 1640 medium (without phenol red) (GIBCO, Invitrogen Co., Paisley, Scotland, UK), containing 10% heat-inactivated fetal bovine serum (GIBCO) and 2.5% 1 M Hepes (GIBCO). Cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2 in air. Resistant cells were maintained without drug for two weeks prior to each experiment.

Exposure of cells to cytostatic drugs. Cells were suspended in pre-heated medium (37˚C) in culture flasks at a concentration of 5×10^5 cells/ml and a final volume of 8 ml. Each cell line was exposed to a final concentration of either 0.2 μM DNR (Cerubidin; Aventis Pharma, Bromma, Sweden), 0.5 μM Ara-C (Cytarabine; Pfizer AB, Sollentuna, Sweden) or RPMI 1640 as a negative control, in three separate experiments. These concentrations were chosen to mimic clinically relevant exposure concentrations (12). The reaction was stopped with 10 ml of ice-cold RPMI 1640. Cells were collected by centrifuging at 400 × *g* for 5 min at 4˚C and the supernatant discarded. For each experiment, samples were collected before drug exposure and after 2 and 16 hours of drug exposure. Incubations were performed twice, each time in duplicate. From the first incubation, first-strand cDNA synthesis was carried out twice; hence real-time RT-PCR was performed a total of three times.

RNA preparation and cDNA synthesis. Total RNA was isolated with an RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, RNA from approximately 4×10^6 cells was eluted in 50 μl of RNase-free water and stored at –80˚C until analysed. The total RNA concentration and the quality of the isolated RNA were determined using an Agilent Bioanalyzer 2100 and an RNA 6000 Nano Assay Kit (Agilent Technology, Santa Clara, USA) according to the manufacturer's instructions. The quality of the RNA was determined using the ratio of 28S to 18S. A ratio above 1.5 was used as a cut-off for acceptable quality RNA. For first-strand cDNA synthesis a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used according to the manufacturer's instructions. Briefly, 0.5 μg of total RNA was used to produce first-strand cDNA with dNTP in a final volume of 20 μl in RNase-free water and stored at –20˚C until analyzed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR).

Rreal-time RT-PCR. Real-time RT-PCR was performed using the thermal cycler TaqMan 7500 Fast Real-Time PCR System (Applied Biosystems) and the 7500 Fast Sequence Detection and Absolute Quantification software packages. PCR cycling conditions were: step one, 95˚C for 2 min and step two, 95˚C for 3 s and 60˚C for 30 s (step two repeated 40 times). As an internal control, human placenta DNA was used and as house-keeping gene 18S (Hs99999901_s1 from Applied Biosystems) was used. PCR reactions were performed in 15 μl using the TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and 1.5 μl cDNA. Experiments were carried out in duplicate and the mean values used for analysis.

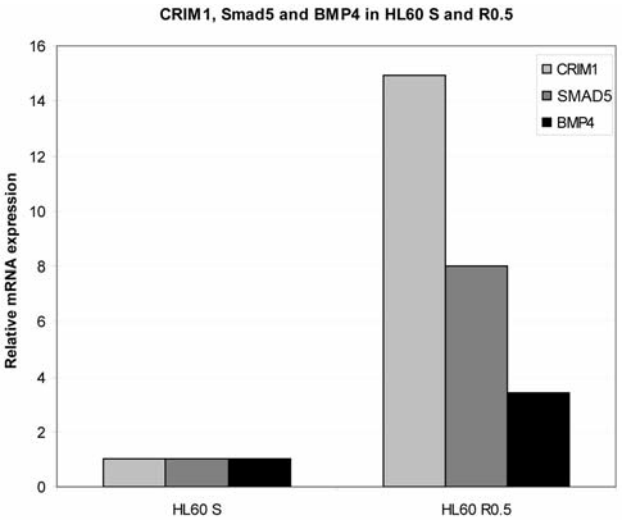

Figure 1. *CRIM1, SMAD5 and BMP4 mRNA levels in drug-sensitive and resistant cell lines. When baseline CRIM1, SMAD5 and BMP4 mRNA levels were set to 1 in drug sensitive HL60S cells, CRIM1 mRNA levels before drug exposure were 15-fold higher in drug-resistant HL60 R0.5 cells. SMAD5 mRNA levels were 8-fold higher in drug-resistant HL60 R0.5 cells. BMP4 mRNA levels in HL60 R0.5 before drug exposure were 3.4-fold higher compared to HL60S.*

Immunofluorescence. Drug sensitive and drug-resistant HL60 cells, at a concentration of 5×10^4 cells in 50 μl, were allowed to dry on glass cover slips, in air at room temperature, overnight. When dry, cells were fixed with cold methanol (–10˚C), and air dried for 5 min at room temperature. After rinsing three times in PBS with 0.1% saponin (Sigma Aldrich, St. Louis, MO, USA) cells were washed twice in PBS, each time for 5 min. To minimize nonspecific binding, all samples were blocked for 30 min in 1.5% bovine serum albumin (BSA) blocking solution (Sigma Aldrich) diluted in PBS plus 0.1% saponin. Samples were incubated with a primary polyclonal rabbit antibody against CRIM1 (Atlas antibodies, Stockholm, Sweden) overnight at 4˚C, at a dilution of 1:200 in PBS plus 1.5% BSA blocking solution. After washing 3×5 min with PBS, samples were incubated with a FITC-conjugated secondary antibody (1 μg/ml) (Dako, Glostrup, Denmark) diluted in PBS with 1.5% BSA blocking solution for 30 min. The cells were counter stained using DAPI mixed with mounting media and the slides were mounted. Samples were analyzed using a Leica Confocal Systems Microscope equipped with a Leica confocal system (Leica Microsystems, Heidelberg, GmbH, Germany).

Results

HL60 CRIM1 mRNA levels. CRIM1 mRNA expression levels in drug-resistant HL60 cells were determined and compared to CRIM1 mRNA levels in drug sensitive HL60 cells. The results show that the CRIM1 mRNA levels were

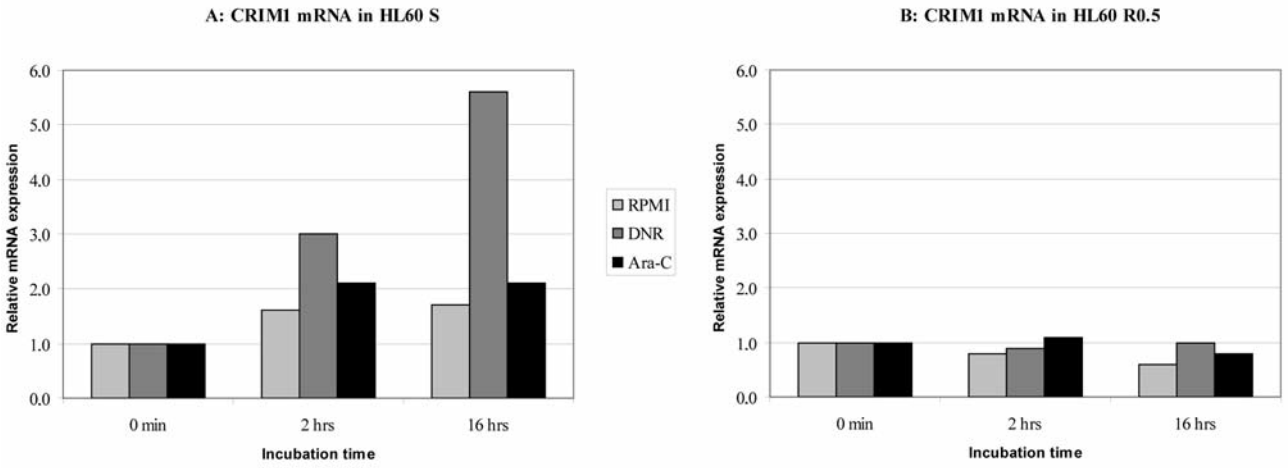

Figure 2. *A: CRIM1 mRNA levels in drug-sensitive cells. Culturing of drug-sensitive HL60S cells in the absence of drugs resulted in a 1.6-fold increase in CRIM1 mRNA levels, when baseline levels were set to 1. After exposure to daunorubicin or ara-C for 2 hours, CRIM1 mRNA levels increased 3- and 2-fold, respectively. After 16 hours of exposure to daunorubicin, CRIM1 mRNA levels increased 5.6-fold, whereas 16 hours of exposure to ara-C did not result in any further increase. B: CRIM1 mRNA levels in drug-resistant cells. When baseline levels were set to 1, CRIM1 mRNA levels in drug-resistant HL60 R0.5 cells cultured in the absence of drugs were 62% lower at 16 hours compared to baseline levels at the start of culturing. Exposure of drug-resistant HL60 R0.5 cells only resulted in minor changes in CRIM1 mRNA levels.*

Table I. *Covariance of Pgp and CRIM1 mRNA expression levels in drug resistant HL60 R0.5 cells, when baseline levels in drug sensitive HL60 S cells were set to 1.*

	Relative mRNA level	
	Pgp	CRIM1
HL60S	1	1
HL60 R0.5	91	15

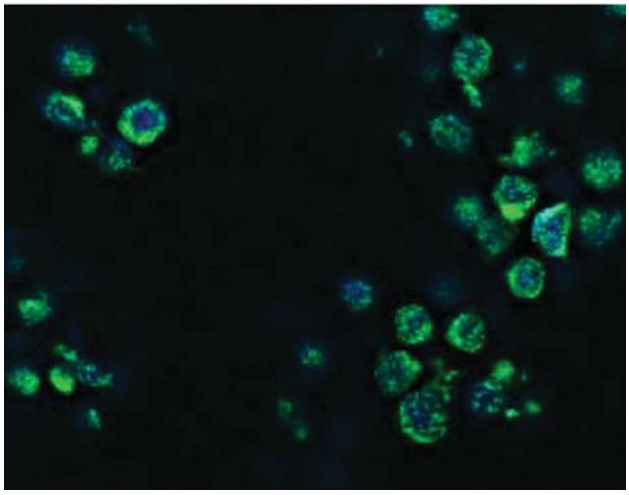

Figure 3. *CRIM1 localization in drug-resistant cells. Simultaneous staining of drug-resistant HL60 R0.5 cells with FITC-conjugated CRIM1 antibody (green) and DAPI (blue), confirms that CRIM1 is located at the membrane surface of myeloid leukemia HL60 R0.5 cells.*

approximately 15-fold higher in drug-resistant cells compared to drug-sensitive cells (Figure 1). After exposure to DNR or Ara-C for 2 hours, CRIM1 mRNA levels in drug-sensitive cells increased 3- and 2-fold, respectively. After 16 hours of exposure to DNR, CRIM1 mRNA levels increased 5.6-fold, whereas 16 hours of exposure to Ara-C did not result in any further increase in CRIM1 mRNA levels in drug-sensitive cells. In drug-resistant cells cultured in the absence of drugs, CRIM1 mRNA levels were 62% lower at 16 hours compared to baseline CRIM1 mRNA levels at the start of the culture (at time 0 hours). Exposure of drug-resistant cells to DNR or Ara-C resulted in no effect on CRIM1 mRNA levels (Figure 2A and 2B).

HL60 SMAD5 mRNA levels. SMAD5 mRNA levels in drug-resistant HL60 cells were determined and compared to drug-sensitive HL60 cells. The pattern of SMAD5 mRNA expression levels was found to resemble that for CRIM1 mRNA levels in both drug-sensitive and drug-resistant HL60 cells. The baseline level of SMAD5 mRNA was 8-fold higher in drug-resistant cells than in drug-sensitive cells prior to drug exposure (Figure 1). After exposure to DNR or Ara-C for 2 hours, mRNA levels in drug sensitive cells increased 5- and 3-fold, respectively. After 16 hours of exposure to

DNR, SMAD5 mRNA levels increased 8-fold, whereas 16 hours of exposure to Ara-C resulted in a 1.8-fold increase in SMAD5 mRNA.

HL60 BMP mRNA levels. BMP4 mRNA levels in drug-resistant HL60 cells were determined and compared to drug-sensitive HL60 cells. BMP4 mRNA levels before drug exposure were found to be 3.4-fold higher in drug-resistant cells compared to drug sensitive cells (Figure 1). No further increase in BMP4 mRNA levels was seen over time after exposure to DNR or Ara-C of drug-sensitive or drug-resistant cells. Neither drug-sensitive nor drug-resistant HL60 cells expressed detectable levels of BMP7.

HL60 Pgp mRNA levels. The mRNA expression levels of Pgp in drug-sensitive and drug-resistant HL60 cells were determined and compared to mRNA expression levels of CRIM1. When Pgp and CRIM1 mRNA expression levels in sensitive HL60S cells were set to 1, the expression levels in drug-resistant HL60 R0.5 cells were 91-fold and 15-fold higher respectively (Table I).

Protein expression analysis by immunofluorescence. Simultaneous staining with FITC-conjugated CRIM1 antibody and DAPI confirmed that CRIM1 is located at the membrane surface of HL60 cells (Figure 3).

Discussion

This study investigated for the first time the mRNA expression levels of CRIM1 in leukemia HL60 cells with different degrees of drug resistance. In addition, the mRNA levels for proteins downstream of CRIM1, namely SMAD5, BMP4 and BMP7, were investigated. Wild-type drug-sensitive HL60 cells and HL60 R0.5 cells resistant to 0.5 μM doxorubicin were used (11). The results demonstrated that drug-resistant cells express 15-fold higher mRNA levels of CRIM1 than did drug-sensitive cells. These levels were maintained for at least 16 hours. Baseline levels of SMAD5 mRNA were 8 times higher in drug-resistant cells compared to drug sensitive HL60 cells. Unlike CRIM1 mRNA expression levels, SMAD5 mRNA levels decreased over time but remained higher than the expression levels found in drug-sensitive cells. Baseline levels of BMP4 mRNA expression were also higher in drug-resistant cells than in drug-sensitive cells (*i.e.* 3.4-fold higher). Similarly to SMAD5 mRNA levels, BMP4 mRNA levels decreased over time, and became equal to the expression levels found in drug-sensitive cells. This is consistent with the findings of Wilkinson *et al*. who demonstrated that CRIM1 acts as a BMP4 antagonist (4).

It has been shown that BMPs inhibit proliferation and induce differentiation of hematopoietic cells, and that constitutive activation of BMPs causes an increase in the commitment of hematopoietic progenitors to myeloid differentiation (13). Since CRIM1 affects the levels of BMPs and thereby of SMAD5, CRIM1 may be of importance in the regulation of the growth and differentiation of hematopoietic cells.

Previously, the importance of expression of several markers of drug resistance in patient cells and leukemia cell lines was studied (14, 15). In the present study, a covariance between the well-known marker of drug resistance, Pgp, and CRIM1, in both drug-sensitive and drug-resistant cells was demonstrated. This covariance may be an indication of general activation of defense mechanisms in the cell, which may be either initiated by drug exposure or possibly incidental. The fact that CRIM1 levels in unexposed drug-resistant HL60 R0.5 cells were initially 15-fold higher than in drug-sensitive HL60 cells, and the fact that the CRIM1 mRNA levels increased after exposure to cytostatic drugs, suggests that CRIM1 may be an independent mechanism associated with drug resistance.

Larsson *et al*. showed that SMAD5 can be both up- and down-regulated by CRIM1 (9). However, this study also found a covariance in the mRNA expression levels of CRIM1 and SMAD5, suggesting that SMAD5 may either be affected by exposure to cytostatic drugs or that there may be an alternative system for regulating SMAD5 expression levels. However, SMAD signaling in hematopoiesis has been shown to be very context dependent and the regulation of progenitor cells is much more complex *in vivo* than *in vitro* (9).

In this study the mRNA expression levels of CRIM1, SMAD5, BMP4 and BMP7 were analysed and it was demonstrated for the first time that CRIM1 is expressed at higher levels in drug-resistant leukemia cells than in leukemia cells sensitive to cytostatic drugs, suggesting that CRIM1 can be used as a marker of drug resistance. Further studies are needed to elucidate the role of CRIM1 in drug resistance, in particular to determine the corresponding changes in protein levels following changes in mRNA levels.

Acknowledgements

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