Induction of apoptosis increases expression of non-canonical WNT genes in myeloid leukemia cell lines

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Abstract. With the aim of determining the differential expression of WNT and FZD genes, before and after induction of apoptosis in BCR-ABL positive cells, we treated the myeloid cell line K562 and control cell line HL60 with imatinib mesylate and etoposide, and analyzed relative mRNA expression levels of WNT, FZD and sFRP genes under normal and apoptotic conditions by real-time RT-PCR. We observed marked increase in mRNA levels of FZD4, FZD5, FZD7 and WNT5b, correlating with apoptotic activity and independent of the agent or cell line used. Our results suggest the involvement of non-canonical Wnt signaling in executing programmed cell death in myeloid cell lines.

Introduction

Wnt proteins are a large family of secreted, lipid modified, cystein-rich glycoproteins that serve as ligands for the frizzled (Fzd) family of seven-transmembrane receptors (1). Wnt/Fzd signaling activates evolutionary conserved signaling pathways that have crucial roles in development by regulating proliferation, differentiation, migration and cell-cell adhesion, depending on the cellular context. The wide variety of processes that Wnt signaling controls has made it a crucial component of not only development but also adult homeostasis. Ligand-receptor specificity between the identified 19 Wnt and 10 Fzd proteins is largely unknown with limited data on appropriate ligand-receptor combinations activating distinct intracellular signaling pathways (1,2). Wnt signaling can be classified as canonical and non-canonical pathways, where the canonical pathway is responsible for preventing the degradation of ß-catenin. Wnt-frizzled receptor coupling results in the stabilization and nuclear translocation of ß-catenin. Accumulation of ß-catenin in the nucleus leads to the transcriptional activation of multiple target genes such as c-myc and cyclin D. The presence or exclusion of co-receptors (low density lipoprotein receptor-related protein 5 and 6-LRP5/6) or secreted Wnt antagonists (soluble frizzled related proteins - sFRPs- and Dickkopf proteins) increases the complexity of the molecular mechanisms underlying cellular outcomes.

Non-canonical pathway activation is independent of ß-catenin. Two non-canonical pathways have also been defined: the planar cell polarity (PCP) and the Wnt/Ca^{2+} pathways (3-6). There is an overlap of proposed Wnt and Fzd proteins and secondary messengers participating in these non-canonical pathways, complicating further the interpretation of experimental data on regulation, ligand-receptor partners, and intermediate members of non-canonical signaling. Although our understanding of non-canonical signaling, especially during adult homeostasis is limited; it has been proposed by different authors with supporting experimental data that non-canonical signaling antagonizes the canonical/ß-catenin pathway by exerting signals with opposite outcomes (7,8).

Wnt molecules have been grouped as canonical and non-canonical pathway activators. However this classification is not a clear-cut distinction. There are reports showing that a given Wnt protein may activate different signaling pathways depending on the cellular context, binding specificities to frizzled receptors, experimental conditions and the presence, absence or pattern of co-receptors available (9-11). In short the ability of a given Wnt molecule to trigger canonical or non-canonical signaling is not absolute (9).

Deregulated Wnt signaling has been implied in a variety of human diseases including cancer (12). These studies are mainly limited to the canonical pathway where known proto-oncogene and tumor suppressor gene products function as intermediate signal transducers. There are numerous reports linking ß-catenin stabilization with different forms of solid tumors as well as leukemia. Aberrant Wnt signaling has been reported in a number of leukemia of both lymphoid and myeloid lineages (13). Nature of leukemias differ from solid tumors in that the hematopoietic system of the adult organism is composed from cells generally with a short life span which are renewed by differentiating from a small population of hematopoietic stem cells (HSCs). Many leukemias are either identified or associated with specific chromosomal translocations resulting in oncogenic fusion products that have been shown to block differentiation and/or escape apoptosis and/or induce proliferation or expansion of the transformed cell population. There is growing evidence suggesting Wnt

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signaling plays an important role in normal HSC renewal and that dysregulation of this pathway may lead to leukemia (13).

Chronic myeloid leukemia (CML) is of interest because the initial transformation event occurs in an HSC. The Philadelphia chromosome, der(22)t(9;22)(q34;q11), is the hallmark of CML resulting in the Bcr-Abl chimeric oncogene. Reports on the role of Wnt signaling in HSC renewal, ß-catenin accumulation and canonical pathway activation in CML and the Bcr protein defined as a negative regulator of Wnt signaling, together has provided a line of evidence suggesting deregulation of the pathway may have a role in disease pathogenesis (13,14). BCR-ABL positive cells are shown to be resistant to apoptosis, a feature thought to contribute to the expansion of the malignant cell clone (15). How Wnt signal transduction interacts with apoptotic signaling is a less explored area of research and interestingly there is scarce evidence that Wnt proteins are directly involved in programmed cell death, a crucial process of development. In light of the previously reported ß-catenin accumulation and canonical pathway activation, along with reported resistance to apoptosis of the malignant clone in CML; we aimed to determine the differential expression of genes involved in Wnt signaling, before and after induction of apoptosis in BCR-ABL positive cells.

Materials and methods

Cell lines and chemicals. K652 is a BCR/ABL positive cell line derived from a CML patient in erythroid blast crisis. HL60 is a BCR/ABL negative cell line derived from an acute promyeloblastic leukemia (APL) patient. HL60 was used as a BCR/ABL negative myeloid leukemia cell line control in the apoptotic induction experiments. Both cell lines were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) and grown in RPMI-1640 medium supplemented with 15% FBS and 1% L-glutamine; in a 5% CO_2 saturated incubator at 37˚C.

Imatinib was kindly provided by Novartis Company (Basel, Switzerland). K562 cells seeded at a $5x10^4$ cells/well density in 96-well plates and were treated with imatinib at 2-, 5- and 10-μM concentrations. Caspase 3/7 activation was evaluated by a caspase-3/7 luminometric assay (see below). No significant difference of caspase activation was observed between 5- and 10-μM concentrations and 5 μM imanitib was used in subsequent experiments where mRNA levels were analyzed. The same concentration was used in evaluating gene expression in the HL60 cell line.

Etoposide (Sigma-Aldrich) is a topoisomerase II inhibitor that causes double strand breaks in DNA. To serve as an experimental control, our goal was to define a concentration that has minimal effects on K562 cells but produces a profound apoptotic effect in HL60 cells. Ten, 30, 50 and 100 μg/ml etoposide treated cells where evaluated for caspase activity in both cell lines where 30-μg/ml concentration was found to give the best reproducible results in inducing caspase activity in HL60 but not K562 cells.

Total RNA extraction and cDNA synthesis. In untreated K562 cells, total RNA was extracted for the purpose of determining the mRNA expression of WNT, FZD, sFRP and Cyclin D genes. In treated cells, K562 and HL60 cells were seeded $1.5x10^6$ cells/ml in serum-free, phenol red-free medium in 25 cm^2 cell culture flasks. After 24 h, 5 μM imatinib or 30 μg/ml etoposide was added to each flask and incubated for 6, 12 and 24 h. Cells were harvest and total RNA was extracted using RNeasy® Mini kit (Qiagen). To avoid genomic DNA contamination, RNA samples were treated with RNase-free DNase I (Qiagen) following manufacturer's instructions. RNA integrity and purity were verified by electrophoresis and OD_{260}/OD_{280} nm absorption ratio respectively. cDNA was synthesized by using SuperScript™ First Strand kit (Invitrogen), 2 μg of total RNA and random primers according to the manufacturer's instructions.

Real-time RT-PCR. Real-time PCR was carried out using a Light Cycler® 2.0 (Roche Diagnostics) instrument and Light Cycler FastStart DNA MasterPLUS SYBR Green I (Roche Diagnostics) kit. Reactions were performed in a 20-μl volume with 5 pmol of each primer and 5 μl of cDNA template derived from reverse transcribed RNA of untreated, 6, 12 and 24 h treated cells. ß-actin gene was used as endogenous control and reference gene for relative quantifications. Primer sequences are given in Table I. The same thermal profile was optimized for all primers: a pre-incubation for 10 min at 95˚C, followed by 40 amplification cycles of denaturation at 95˚C for 10 sec, primer annealing at 59˚C for 5 sec, and primer extension at 72˚C for 10 sec. H_2O was included as a no template control. Melting curves were derived after 40 cycles by a denaturation step at 95˚C for 10 sec, followed by annealing at 65˚C for 15 sec and a temperature rise to 95˚C with a heating rate of 0.1˚C per second and continuous fluorescence measurement. Final cooling was performed at 40˚C for 30 sec. Melting curve analyses of each sample were done using LightCycler Software version 4.0.0.23 (Roche Diagnostics). The analysis step of relative quantification is a fully automated process done by the software, with the efficiency set at 2 and the cDNA of untreated cells defined as the calibrator. All samples in glass capillaries were later run in 2% agarose electrophoresis to check for size and non-specific amplifications. All experiments were done in triplicates.

Measurement of caspase-3 activity. Caspase activation was determined by detecting caspase-3 activity in cell lysates using the Caspase-Glo 3/7 Assay Kit (Promega) following the manufacturer's instructions. Briefly cells were washed with PBS, and suspended in serum-free, phenol red-free medium to reduce background readings observed in flouro-metric/luminometric assays in the presence of serum and/or phenol-red. K562 and HL60 cells were seeded in 96-well plates at a density of $5x10^4$ cells/well. After a 24-h incubation, 5 μM imatinib mesylate was added to each well and cells were incubated for 6, 12 and 24 h. Caspase-Glo 3/7 reagent (100 μl) was then added to each well including medium alone and untreated control cells. The reactions were incubated at 37˚C for 45 min, and plates were mixed on a plate shaker for 30 sec before and after incubation. The cleavage of the substrate by caspase 3/7 was detected by using a Synergy HT Multidetection Plate Reader (Biotek Instruments). After subtracting readings for the blank controls, the experimental readings were normalized to control readings of untreated

Table I. List of primer sequences used in RT-PCR experiments [sequences designated with an asterisk (*) were previously reported (25)].

Gene	Sequence	Size	Gene	Sequence	Size
Fzd1F	ACGGCGAACGGGGCATCTCC	407	Wnt6F	CAGCCACAGCAAGGCCTTTG	779
Fzd1R	TGGGGTGCCTTTGTCGGACG		Wnt6R	AGCACCAGTGGAAGCGGCAC	
Fzd2F	CCCTGCCCCGCCTGCTGCTG	474	Wnt7aF	GTCTACCTCGGGATCGGTGG	442
Fzd2R	GTAGCGCAGGAGCTCCGTCC		Wnt7aR	AGACCTTGGCGAAGCCGATG	
Fzd3F	GTGTGTTTTGTCGGCCTCTACG	313	Wnt10aF	TTCTTCCTACTGCTGCTGGCTG	200
Fzd3R	GAATGTGATACTCTCTGCAGCGTTC		Wnt10aR	ATGGCGATCTGGATGCCCTG	
Fzd4F*	TGCCTTTTCAGGGCAAAGTG	400	Wnt10bF	CGGAGCCTCCGGGCTTCGAC	669
Fzd4R*	ACAGGAAGAGATTTATGGAATG		Wnt10bR	TGGAATCCAAGAAATCCCGAG	
Fzd5F*	TACCCAGCCTGTCGCTAAAC	248	Wnt11F	AGGGCGCGGCCGCAGGTCTG	715
Fzd5R*	AAAACCGTCCAAAGATAAACTGC		Wnt11R	GGGTCTTGAGGTCAGCAGCCAC	
Fzd6F	TTTTGGCATCCGATGGCCTG	209	Wnt14F	GACCACCTGCAAGTGCCACG	505
Fzd6R	CACACTGTTCAATTCCCAGAAAC		Wnt14R	ATGCCTGCACCCTGTGCAGC	
Fzd7F*	GTTTGGATGAAAAGATTTCAGGC	294	Wnt15F	GCGAGACCGGCCAGGTGCTG	192
Fzd7R*	GACCACTGCTTGACAAGCACC		Wnt15R	ACTTGCTGGGCCGGCAGAAG	
Fzd8F*	ACAGTGTTGATTGCTATTAGCATG	268	Wnt16F	CGCTGAACAGCCGCCAGAAG	541
Fzd8R*	GTGAAATCTGTGTATCTGACTGC		Wnt16R	ACAGCACAGGAGCCGGAAAC	
Fzd9F*	CCCTAGAGACAGCTGACTAGCAG	263	sFRP1F	CTGTGCCACAACGTGGGCTAC	316
Fzd9R*	CGGGGGTTTATTCCAGTCACAGC		sFRP1R	GCGGGGTCATGGCGATGCAG	
Wnt1F	GGCCCCACCTCTTCGGCAAG	502	sFRP2F	TCCTCGCCTCGCACTGCTGC	372
Wnt1R	GGCTCCAGGCGCAGCAGCTC		sFRP2R	AGGGGAAGCCGAAGGCGGAC	
Wnt2F	GCCATCACCAGGGCCTGTAG	503	sFRP3F	CCTGGCTGCTCTCTGCCTGC	266
Wnt2R	GTACCCAGGGAGCCTGCCTC		sFRP3R	GCTTGATGGGCTCGTGCTGG	
Wnt2bF	GGGGACTTTGACTGGGGTGG	365	sFRP4F	GTCCACATATCCTGCCCCATC	259
Wnt2bR	AAGTAGACAAGATCAGTCCGGGTG		sFRP4R	GGACTGGCAGGTTTGGGAGC	
Wnt3F	GGCACCAGGGCGCTGGGAAG	300	sFRP5F	CGCTGCACGGCCGCTCCTAC	468
Wnt3R	CATGTGCAAAGATAAGCCTCAGG		sFRP5R	TCTGTTCCATGAGGCCGTCAG	
Wnt4F	GGTCAGCCCACAGGGCTTCC	407	Cyclin D1F	GCGAGGACCAGAAGTGCGAGG	241
Wnt4R	AGAAGTCCGGGCTAGGCTCC		Cyclin D1R	AGCTTGTTCACCAGGAGCAGC	
Wnt5aF	TTTCTCCTTCGCCCAGGTTG	321	ß-Actin F	CCTTCCTGGGCATGGAGTCCTG	202
Wnt5aR	GCGTACGTGAAGGCCGTCTC		ß-Actin R	GGAGCAATGATCTTGATCTTC	
Wnt5bF	TTCTCTCCCTCTGGCGAGGAC	550			
Wnt5bR	AGCCTGGTCATGCACCTGG				

cells under the same conditions. Data were analyzed using KC4 v3.4 (Biotek Instruments) software. Results are expressed as the mean ± SD. The same procedure was performed for 30 μg/ml etoposide treated cells. All experiments were done in triplicates to ensure reproducibility.

Flow cytometry for annexin V/propidium iodide (PI). Apoptosis was assessed by staining cells with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI). In brief, cells were washed with PBS, and suspended in serum-free, phenol red-free medium (with the aim of reproducing the

same media conditions in which caspase activity was detected). K562 and HL60 cells were seeded in 6-well plates at a density of $5x10^5$ cells/well. After a 24-h incubation, 5 μM imatinib mesylate was added to each well and cells were incubated for 6, 12 and 24 h. After the incubation period cells were washed twice with cold PBS. The level of annexin V binding was determined by using a commercially available annexin V apoptosis detection kit (Annexin V-FITC Kit, Beckman Coulter, PN IM3546), according to the manufacturer's instructions. The cells were subsequently analyzed by a Coulter Epics L.MCL flow cytometer (BeckmanCoulter Inc. Miami, FL). Approximately 10,000 events were collected for each sample. The percentage distributions were calculated by Expo32 ADC software (Beckman Coulter, Inc., Miami, FL). Cells were classified as apoptotic (positive annexin V and negative PI), late apoptotic/necrotic (positive annexin V and positive PI) or viable (negative annexin V and PI). Unstained samples were used as negative fluorescence controls. The same procedures were performed for 30 μg/ml etoposide treated cells.

Results

We first examined which genes are expressed under normal culture conditions in untreated K562 and HL60 cells. mRNA expression for WNT1, -2, -3, -3a, -4, -5a, -5b, -6, -7a, -9a, -9b, -10a, -10b and -16 were examined. WNT7b, -8a, -8b and -11 were not included in this study. Triplicate experiments were performed and we observed reproducible results in both cell lines for the expression of WNT2b, WNT3, WNT3a, WNT5b and WNT10a genes in all three experiments. FZD1 through FZD9 (FZD10 was not included in this study) were reproducibly shown to be expressed in the K562 cell line. HL60 cells were found to express FZD3 through FZD9, FZD1 and FZD2 mRNA expression were not detected. Of the five human sFRP genes examined, we were able to detect mRNA expression only for sFRP4 in K562 cells; whereas both sFRP1 and sFRP4 mRNA expression were detected in HL60 cells. WNT, FZD and sFRP genes that were expressed under normal culture conditions in untreated K562 and HL60 cells were further examined for differential expression after apoptotic stimuli.

Imatinib mesylate (imatinib) inhibits the kinase activity of the Bcr/Abl fusion protein by occupying the ATP-binding pocket of Abl. Apoptotic induction is presumably a result of blocking survival signals provided by the BCR/ABL fusion product. Imatinib mesylate (5 μM) was added to K562 cells and incubated for 6, 12 and 24 h after which activity of caspase-3 and -7 were determined. Caspase activity of K562 cells markedly increased at 12 and 24 h after treatment with imatinib (Fig. 1). The HL60 cell line was used as a BCR/ABL negative control. As expected we did not observe a significant increase of caspase activity in 5 μM imatinib treated HL60 cells when compared to untreated control cells. We repeated the above experiments by incubating cells with 30 μg/ml etoposide instead of imatinib; a concentration previously determined not to induce apoptosis in K562 cells. No significant increase in caspase activity was detected in etoposide treated K562 cells, when compared to untreated cells. On the contrary a 5-fold increase of caspase activity was observed in etoposide treated HL60 cells by the 6th hour (Fig. 1).

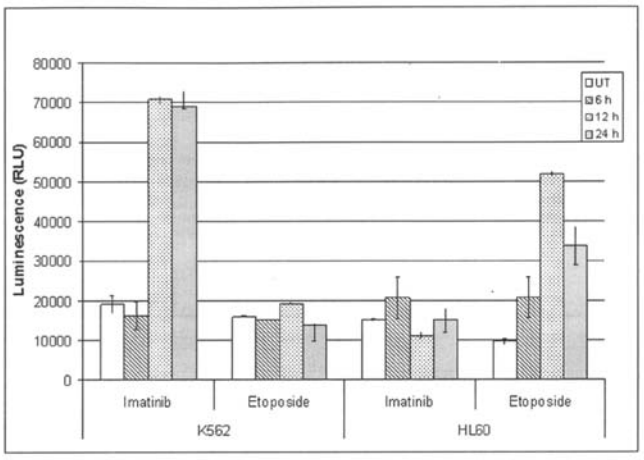

Figure 1. Luminescence readings of the caspase 3/7 assays in K562 and HL60 cells after 5-μM imatinib and 30-μg/ml etoposide treatment (RLU, relative light unites; UT, untreated).

Flow cytometry data for annexin V/PI staining correlates with caspase-3 activation (Fig. 2). A decrease of 15-20% in viability is observed in untreated K562 and HL60 cells, most probably due to the incubation period in serum free media. After 12 h of imatinib treatment, cell viability decreases to 36% in K562 cells, whereas it is approximately 64% in HL60 cells. After 12 h of etoposide treatment, 66% cell viability was observed in K562 cells as opposed to the 32.5% viability observed in the HL60 cell line.

Following imatinib treatment we analyzed the differential mRNA expression of genes previously determined to be expressed in the K562 cell line. We did not detect any significant difference in the relative mRNA expression levels of WNT3, WNT3a, FZD1, FZD3, FZD6, FZD8, FZD9 and sFRP4. Interestingly we observed a time-dependent linear increase in mRNA levels of WNT2b, WNT5b, WNT10a, FZD2, FZD4, FZD5 and FZD7 genes (Fig. 3A) after imatinib treatment. The most striking increase in mRNA expression was observed for FZD4, where a 25-fold increase at 24 h was detected; followed by FZD5 and FZD7 genes both with a 12-fold increase of their expression. Approximately a 5-fold increase in mRNA levels for WNT2b and WNT5b, a 3-fold increase for WNT10a and a 7-fold increase for FZD2 were observed at 24 h.

We analyzed expression levels of the same genes in HL60 cells where imatinib was shown not to have an apoptotic effect. No significant increase in mRNA expression was observed in any of the genes analyzed (Fig. 3B). We concluded that the observed increase in mRNA expression was not a nonspecific effect of imatinib and correlated with apoptotic death of treated cells.

Although the observed increase in mRNA levels correlate with apoptotic cell death of imatinib treated cells, the question remains whether this effect is specific to imatinib-induced cell death or the K562 cell line. We repeated the above experiment by treating K562 cells with 30 μg/ml etoposide instead of imatinib (Fig. 4A). FZD4 expression showed only a 1.5-fold increased at 6 h, whereas no difference in WNT5b

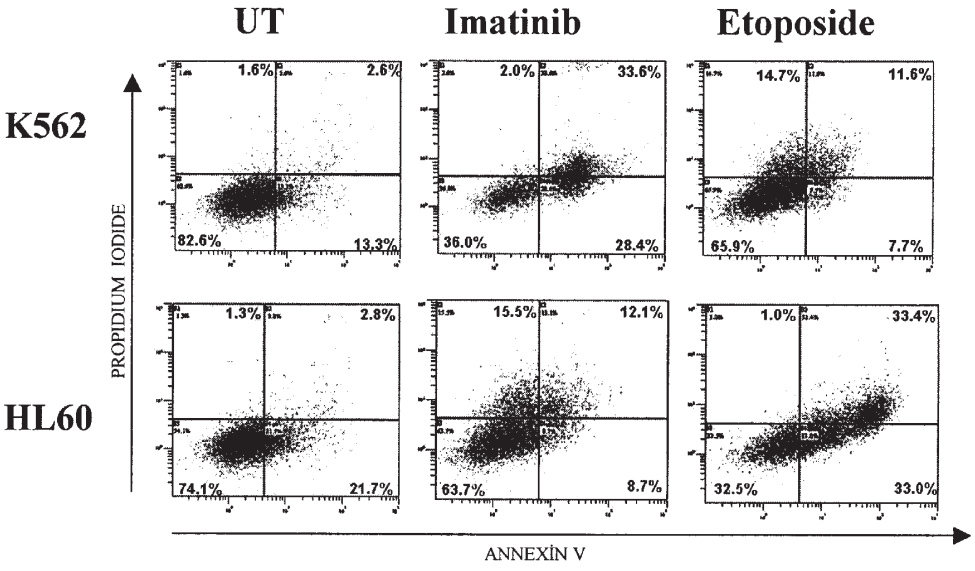

Figure 2. Flow cytometry percentage distributions after annexin V/propidium iodide staining in 5 μM imatinib and 30 μg/ml etoposide treated K562 and HL60 cells (UT, untreated cells).

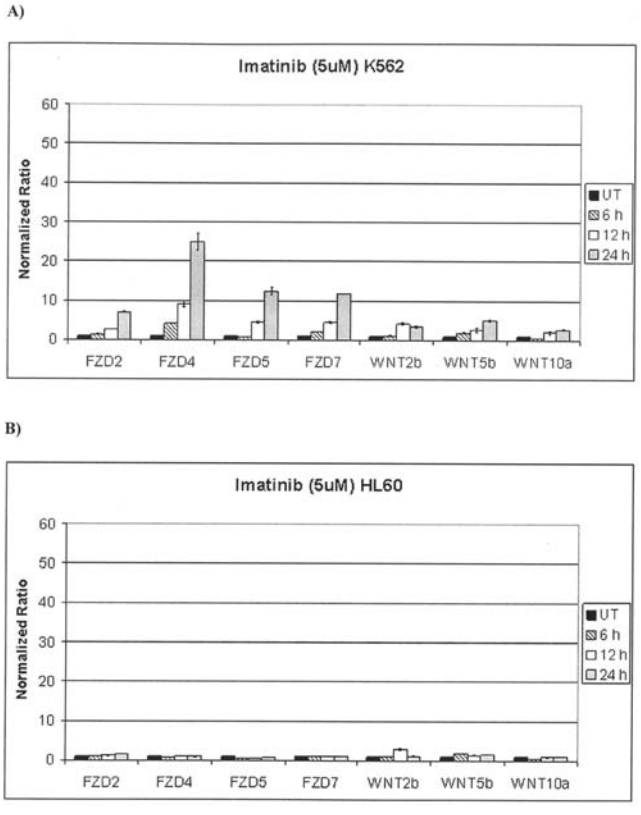

Figure 3. mRNA levels after 5-μM imatinib treatment in: (A), K562; and (B), HL60 cells. Gene expression is first normalized to the reference gene ß-actin, followed by normalization of the sample ratio to the calibrator ratio (UT, untreated cells).

expression was detected. A 5-fold increase of FZD7 expression at 6 h and 2.5-fold increase in FZD5 expression at 6 and 12 h were observed followed by a decline in mRNA levels by 24 h. WNT10a showed a 1.5-fold steady increase of expression at all time-points. Only the expression pattern of WNT2b seemed similar to that of imatinib treated cells with a 5-fold steady increase of mRNA expression.

mRNA levels detected in etoposide-induced apoptotic HL60 cells are in sharp contrast to the expression data of etoposide treated K562 cells (Fig. 4B). There is a striking 55-fold time-dependent increase of FZD4 and a 7-fold increase of WNT5b mRNA expression. A 10-fold increase of WNT2b was detected at 6 h, which rapidly declines back to normal levels at 12 h. A 4-fold increase in FZD5 and a 2.5-fold increase in FZD7 mRNA expression were observed at 6 h, both declining in a time-dependent manner. WNT10a shows a slight increase of 1.5-fold by 24 h, whereas no difference in expression levels was detected for FZD2 when compared to untreated cells.

Constitutive ß-catenin signaling has been reported in various cancers, mainly as providing a proliferation and survival signal. Therefore it does not seem relevant for having a direct contribution in executing cell death. On the contrary one may expect a down regulation of canonical pathway target genes once the cell has committed itself to apoptosis. We examined the mRNA expression levels of a well-established downstream target of the Wnt canonical pathway; the cyclin D1 gene in imatinib and etoposide treated and untreated cells. Cyclin D1 mRNA levels were not significantly different from untreated cells in the imatinib treated HL60 cell line. In imatinib treated K562 cell a marked decrease in cyclin D1 levels nearly 10-fold below untreated cells was observed (Fig. 4C). In etoposide treated HL60 cells cyclin-D1 mRNA levels started to decline by 6 h, reaching a 10-fold decrease by 24 h. We also observed a steady decline in cyclin-D1 mRNA levels in etoposide treated K562 cells reaching 5-fold by 24 h, supporting a cell cycle block due to the DNA assault exerted by etoposide. Although these data may support the antagonizing effect of non-canonical Wnt signaling; it must be interpreted with caution, taking into account that there is cross talk between multiple signaling pathways in the regulation of the cyclin D1 gene.

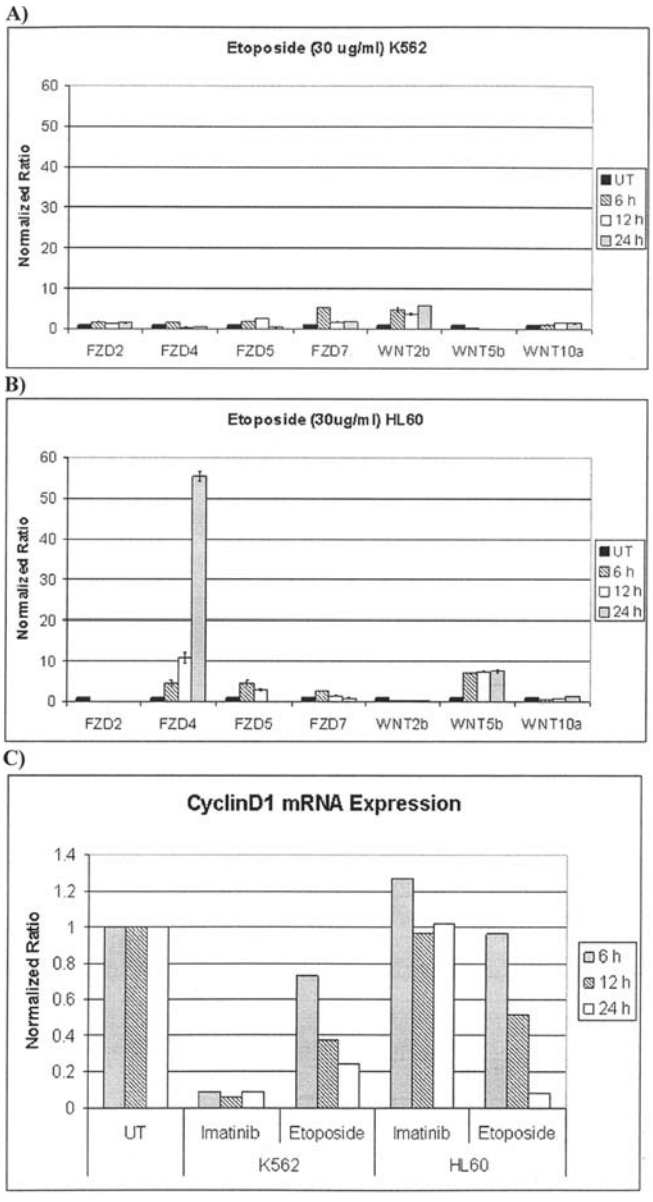

Figure 4. mRNA levels after 30-μg/ml etoposide treatment in: (A), K562; and (B), HL60 cells (UT, untreated). (C), Cyclin-D1 mRNA levels after 5-μM imatinib and 30-μg/ml etoposide treatment in K562 and HL60 cells (UT; untreated).

Discussion

Resistance to apoptosis is a documented feature of BCR-ABL positive cells in CML. Recent reports have shown ß-catenin accumulation and canonical pathway activation in CML, suggesting deregulation of the pathway may have a role in disease pathogenesis (13,16). In addition, the Bcr protein which is disrupted as a result of the t(9;22)(q34;q11) translocation in CML, has been reported to function as a negative regulator of Wnt signaling (14). In light of these recent data we aimed to analyze the differential mRNA expression of genes involved in Wnt signaling before and after induction of programmed cell death. We treated the myeloid cell lines K562 and HL60 (control cell line) with imatinib and etoposide, two agents that exert their apoptotic effect by different and distinct mechanisms. We analyzed the differential mRNA expression of WNT, FZD and sFRP genes under normal and apoptotic conditions by real-time RT-PCR. We observed marked increase correlated with apoptotic activity in the mRNA levels of FZD4, FZD5, FZD7 and WNT5b genes, independent of the agent or cell line used.

Our initial expectation was to observe a down-regulation of genes that code products functioning in the canonical Wnt pathway previously reported to be activated in CML. Unexpectedly, upon induction of apoptosis an up-regulation of genes previously defined in non-canonical Wnt signaling is observed. Our results suggest that the observed increase of mRNA expression correlates with apoptotic activity in myeloid leukemia cell lines and is neither apoptotic agent nor cell line specific. Although unexpected, these observations support the view that non-canonical signaling may be acting as an antagonist of the canonical Wnt pathway, which promotes cell survival and proliferation (7,8).

The increase of FZD4 mRNA levels was profound in both cell lines, even though different compounds were used for apoptotic induction. Fzd4 has been reported to function as a receptor in non-canonical Wnt pathways where it was shown to activate protein kinase C (PKC) (17). The PKC family consists of 12 isozymes that may have inhibitory or stimulatory effects on apoptosis depending on the signaling context (18). The PKCδ isozyme is frequently associated with pro-apoptotic stimulation. The pro-apoptotic effects of PKCδ is suggested to exert its influence by inhibiting c-Jun N-terminal kinase (JNK) activity (18), a kinase proposed to function downstream of the PCP pathway. In addition, over-expression of Dickkopf-1, an antagonist of the canonical pathway, is reported to induce apoptosis through JNK in a human choriocarcinoma cell line (19). In light of these previous reports it is possible that Fzd4 may be involved in the induction of apoptosis by interacting with PKC signaling. WNT5b, another gene in which we observed marked increased of mRNA levels, is also a non-canonical Wnt implicated in the PCP pathway and induction of JNK signaling (3,8). Interstingly, Kanazawa *et al* have reported a decrease in the expression of several anti-apoptotic genes such as insulin-like growth factor 1 (Igf-1) and Wnt1-inducible secreting protein 1 (Wisp1) in Wnt5b over-expressing 3T3-L1 cells; further supporting a role for Wnt5b in programmed cell death (20). Further research is needed to verify the relevance of the observed increase mRNA expression in these genes and the hypothetical link between FZD4, Wnt5b, PKC and apoptosis.

We also observed increases in WNT2b, FZD5 and FZD7 mRNA levels in both imatinib treated K562, etoposide treated HL60 cells and to a lesser extent etoposide treated K562 cells, although the pattern and level of increase was different in each experiment. WNT2b, FZD5 and FZD7 are reported to predominantly function in canonical Wnt signaling (3,21), although there are also a few reports implying a role for Fzd7 in non-canonical signaling (22,23). It is tempting to speculate that the increase in mRNA levels observed for WNT2b, FZD5 and FZD7 might correlate with DNA damage; since DNA cleavage is a late event in imatinib induced programmed cell death; whereas it is the primary cause of apoptosis in etoposide treatment.

Although an increase in FZD2 mRNA level was observed in imatinib treated K562, no difference in expression levels was detectable in etoposide treated HL60 cells. FZD2 was found to be expressed at considerably lower levels in untreated HL60 when compared to the untreated K562 cell line. We proposed that the observed discrepancy in mRNA levels after apoptotic induction might be a result of the different biological background of the cell lines used.

There was also a slight increase WNT10a in apoptosis induced myeloid cell lines. Over-expression has been shown in several tumor types and cancer cell lines supporting the view of a potential role in B-catenin signaling (24), but the limited number of reports can not establish its function.

In conclusion, we have shown that mRNA expression of FZD4, FZD5, FZD7, WNT5b and to a lesser extent WNT10a genes are up-regulated in response to apoptotic stimuli, independent of the cell line or agent used. The correlation between the increased expression of non-canonical Wnt genes and apoptotic stimulation is a novel observation suggesting the possible involvement of the non-canonical Wnt pathway in executing programmed cell death, adding another layer of complexity on interactions between Wnt- and other signaling cascades.

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