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G2/M Arrest Sensitises Erythroid Leukemia Cells to TRAIL-induced Apoptosis

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Abstract

Erythroidl eukemia is a heterogeneous disease with very poor prognosis. It may arise de novo, secondary to myelodysplastic syndrome, blast crisis phase of chronic myeloid leukemia, or after cytotoxic therapy of acute myeloid leukemia. The current mainstream treatment of erythroleukemia is cytarabine and anthracyclin-based chemotherapy or bone marrow transplantation. In the current study we found that cytarabine or inhibition of the DNA-damage-activated protein kinase, ATM, induce G2/M arrest and sensitised K562 erythro leukemia cells to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL). Arresting cells in G2/M with microtubule-disrupting drugs also enhanced TRAIL-sensitivity. Synchronisation or separation of the leukemia cells in different stages of the cell cycle by elutriation confirmed that the cells in G1 and G2/M were sensitive to TRAIL. Interestingly, this sensitivity was associated with cell cycle-dependent oscillation of cFLIP expression. In summary, we found that combination of cytostatic drugs with TRAIL can be an effective treatment for erythroid leukemia.

Keywords: TRAIL; Apoptosis; Combination therapy; DNA damage response; Cell cycle; Erythroid leukemia

Introduction

TNF-related apoptosis inducing ligand (TRAIL/APO2L) is a death ligand member of the TNF cytokine super family that interacts with five different receptors, two of which can initiate apoptosis (death receptors DR4 and DR5), whereas the other three are regulatory or decoy receptors (DcR1, DcR2 and osteoprotegerin (OPG)). Binding of TRAIL to DR4 or DR5 results in receptor activation followed by sequential recruitment of the adaptor protein Fas-associated death domain (FADD) and pro-caspase-8, thus forming the death-inducing signalling complex (DISC). At the DISC, pro-caspase-8 is auto activated and initiates a caspase cascade resulting in dismantling of cellular components and consequent cell death [1]. TRAIL is highly selective to tumour cells and unlike many other apoptotic pathways, the it is not affected by p53-deficiency [2]. Therefore there is significant interest in TRAIL as a potential anti-cancer therapeutic.

Acute erythroidleukemia (AEL) is a rare form of acute myeloid leukemia (AML) with generally poor prognosis. AEL is a heterogeneous disease often driven by highly undifferentiated myelogenic progenitor cells characterised by genetic instability and poor prognosis with the erythroidleukemia (pEL) subtype having a patient median survival of 3 ± 3.6 months [3].

The mainstream therapy for erythroid leukemia is either bone marrow transplantation or genotoxic drug therapy (cytarabine/daunorubicin combination). Cytarabine (araC) competes with cytidine for incorporation into the DNA leading to inhibition of DNA polymerases and consequent disruption of DNA synthesis, termination of chain elongation that causes DNA damage [4].

The generally low sensitivity of leukemic cells to TRAIL makes it a less attractive therapeutic for these cancers [5]. Recent studies however identified drug combination strategies that could potently sensitize TRAIL-resistant tumours [6]. Additionally, a recent study on acute lymphocytic leukemia has shown that the undifferentiated leukemia-initiating cell (LIC) population is sensitive to TRAIL and tumour extracts from TRAIL-treated xenografts fail to re-establish the tumour when transplanted into a new animal [7] highlighting the possible potential of TRAIL for targeting the undifferentiated cells driving erythroid leukemia.

Based on these findings the aim of the current project was to test whether a TRAIL-based therapy is able to eradicate erythroid leukemia cells. We found that K562 erythroleukemia cells are partially sensitive to TRAIL and araC and TRAIL act synergistically. Furthermore, we found that TRAIL sensitivity is linked to normal cell cycle progression as cells in early G1 or G2/M phase isolated by elutriation displayed higher TRAIL-sensitivity, which correlated with a cell cycle-dependent, oscillating expression of the anti-apoptotic protein cFLIP. Arresting the cells in G2/M by either araC, inhibition of ATM or microtubule disruption further increased TRAIL-sensitivity. In conclusion, these results indicate that arresting erythroid leukemia cells in G1 or G2/M using cytostatic or genotoxic drugs can render them sensitive to TRAIL offering a potential novel treatment avenue.

Materials and Methods

Cellculture and treatments

The K562 erythroleukemia cell line derived from blast crisis chronic myelogenous leukemia and positive for Bcr-Abl(CML)(ATCC # CCL-243™) [8], was cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum, 2 mM glutamine, 1 mM pyruvate, 50

U/ml penicillin and 50 μM streptomycin (all from Sigma) and maintained at 37°C, 5% CO_2 in a humidified incubator.

Cells were seeded at 2x105 cells/ml in 24-well plates 24 h before treatment. Cytarabine (araC) (C1768-Sigma) was resuspended in water at 100 mM and used at indicated concentrations for individual experiments. TRAIL was produced according to [9]. Inhibitors of DNA-PK (NU7441), ATM (KU55933) and Chk1 (UCN-01) were purchased from Axum, BioConmed and Sigma, respectively. Inhibitors were resuspended in DMSO and were used at the following concentrations: 10 μM for NU-7441, 10 μM for KU-55933 and 100 nM for UCN-01. Control cells were treated with equivalent volumes of vehicle.

Annexin V staining

1x105 cells collected by centrifugation, resuspended in 50 μl calcium buffer (10 mM HEPES pH7.5, 140 mM NaCl, 2.5 mM $CaCl_2$) containing 0.5 μl Annexin V-FITC and incubated for 15 min incubation on ice in the dark before adding 300 μl of calcium buffer and acquired using a FACSCanto II flow cytometer (BD Biosciences).

Propidiumidodide (PI) staining

3x105 cells were fixed in 70% ethanol in PBS and stored at -20. Cells were centrifuged at 3,000 RPM for 7 min then resuspended in Hanks' solution and incubated at 37 for 30 min before recollecting them (10,000 RPM, 5 min), and treating with RNase-PI (Amersham) for 15 min and acquired using a FACSCanto II flow cytometer and analysed using Diva™ software. Cell death induction was determined as the percentage of cells in sub-G1. Specific apoptosis was calculated as (% apoptotic cells with treatment - % apoptotic cells without treatment) / (100 - % apoptotic cells without treatment) x 100, to correct for the presence of dead cells in the untreated samples. Cell cycle distribution was determined from the cycling population.

Assessment of cell morphology (apoptotic index)

Cell morphology has been studied and quantified on haematoxylin and eosin-stained cytospins as described before [10].

Induction of mitotic arrest

Cells were treated with 0.3 μM of the microtubule inhibitors colcemid and nocodazole (in DMSO; Supplementary Figure 3) for 16 h before treatment with TRAIL. Alternatively, cells were washed twice after nocodazole treatment and either treated immediately or released for 6h to generate cells in G1 phase prior to TRAIL treatment.

Western blot analysis

Cells were harvested by centrifugation, lysed, denatured and proteins separated and blotted as described before [6]. Blots were incubated with rabbit polyclonal antibodies against caspase-3 (1:1 000; Cell Signaling Technologies, Danvers, MA, USA), Mcl-1 (1:1 000; Cell Signaling Technologies), Cyclin E (1:1 000, Sigma), Cyclin B1 (1:1 000,ThermoScientific),or actin (1:500; Sigma) and mouse monoclonal antibodies against caspase-8 1C12 (1:1 000; Cell Signaling Technology), XIAP (1:5 000; Assay Design), cFLIP (1:200 Alexis Pharmaceutical), Bcl-XL (H-5) (1:200; Santa Cruz) and Bcl-2 (100) (1:200; Santa Cruz).For detection, appropriate horseradish peroxidase-conjugated goat secondary antibodies were used. Protein bands were visualized with SuperSignal® West Pico Chemiluminescent Substrate (Pierce) or Immobilon western HRP substrate (Millipore) on X-ray film (Agfa).

Centrifugal elutriation

Enrichment of K562 cells in the different phases of the cell cycle was achieved using a J-26 XP Beckman elutriation centrifuge with a JE-5.0 rotor equipped with a single-standard 5 ml elutriation chamber (Beckman Coulter, Inc., Fullerton, CA, USA) and a Masterflex®L/S®peristaltic pump, model 7523-47 (Cole Parmer). Rotor speed was maintained at 2,200 RPM at 8°C, and the medium flow-rate was controlled by a Cole-ParmerMasterflex pump. Cells were equilibrated in the chamber with a constant flow rate of 15 ml/min. 100 ml fractions were collected at flow rates increasing from 15 to 60 ml/min. PI-stained samples from each fraction were analysed by flow cytometry to determine the enrichment. Cell fractions were seeded at $3x10^5$ cells/ml in 24-well plates and treated with TRAIL immediately. Statistical analysis was carried out using two-way Anova with GraphPad Prism.

Statistical analysis

All results presented are the outcome of the minimum of three independent repeats. Significant differences have been determined using paired student t-test with a $p<0.05$ significance threshold.

Results

AraC sensitises K562 cells to TRAIL-induced apoptosis

K562 cells were treated for 24 h or 48 h with 1-5 μM of araC and induction of cell death measured. Flow cytometric analysis of the sub-G1 population and AnnexinV staining showed that araC induced up to 27% apoptosis (Supplementary Figure 1A-1C). Cell cycle analysis showed that araC decreased the percentage of K562 cells in S phase and increased the proportion of cells in G2/M consistent with a delay in S phase progression, and G2/M arrest (Supplementary Figure 1D).

To determine whether the combination of araC and TRAIL have a stronger cytotoxic effect, cells were treated with a dosage of araC (1-5 μM) for 24 h, followed by treatment with 250 ng/ml of TRAIL for an additional 24 h (Figure 1A and 1B). TRAIL (250 ng/ml) alone induced a maximum of 30% apoptosis determined with PI staining (Figure 1A and 1B) or AnnexinV (E), while pre-treatment with araC resulted in a dose-dependent increase in TRAIL-induced sub-G1 population (from 32.3 ± 4.8 to 64.0 ± 3.1%; Figure 1A and 1B). Similar results were gained with AnnexinV staining (Supplementary Figure 1E). Flow cytometric analysis of cell cycle distribution revealed that the proportion of cells in G2/M phase decreased after combined araC/TRAIL treatment (Figure 1C) suggesting that cells in G2/M were being depleted by the combination treatment.

DDR kinase inhibition sensitises K562 cells to TRAIL

The cell cycle arrest observed after araC treatment suggests DDR activation. To characterise the effect of DDR protein kinase inhibition on TRAIL-induced apoptosis we used specific inhibitors of DNA-PK (NU7441) [11], ATM (KU55933) [12], and Chk1 (UCN-01) [13,14]. Treatment of cells with NU7441 and UCN-01 alone led to a slight increase of apoptosis as determined by PI staining (Figure 2A), while KU5933 alone induced apoptosis in 20.8 ± 3.1% of the cells (Figure 2A). When TRAIL was combined with the DDR kinase inhibitors,

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DNA-PK inhibition or Chk1 inhibition resulted in a reduction of TRAIL-induced apoptosis, while ATM inhibition enhanced it by two-fold (Figure 2A). Comparable results were obtained after Annexin V staining (Supplementary Figure 2).

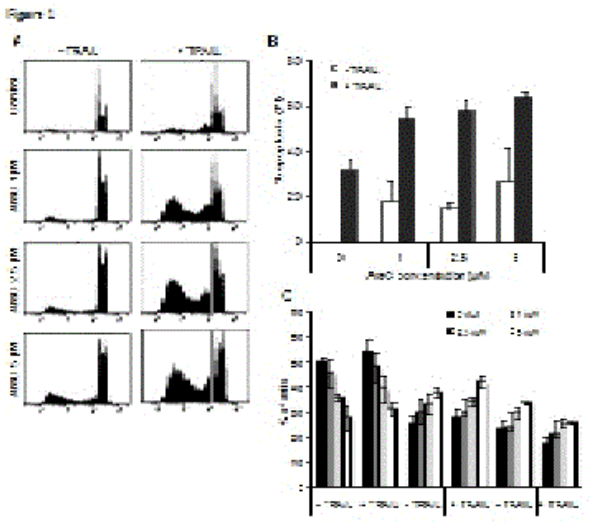

Figure 1: AraC sensitizes K562 cells to TRAIL-induced apoptosis. K562 cells were treated for 24h with the indicated doses of cytarabine (araC) followed by 24 h treatment with 250 ng/ml TRAIL, before being harvested for PI staining. Analysis of PI by flow cytometry was used to determine the percentage of Sub-G1 cells in the cycling population. A) Flow cytometry histograms from a representative experiment for determination of the percentage of Sub-G1 cells after combination treatment with araC and TRAIL. B) Effect of combination treatment of araC and TRAIL on apoptosis induction. Data represent the average % of cell death induced ± STD. C) Effect of combination treatment with araC and TRAIL on cell cycle distribution. Data represent the mean percentage of cells ± STD.

Cell cycle analysis showed that NU7441 increased the percentage of cells in G1, and a decrease in S phase, while inhibition of Chk1 led to an increase in S phase and a decrease in G2/M. Administration of TRAIL did not alter this trend (Figure 2B). The effect of KU5933 on cell cycle distribution was more pronounced (Figure 2B). It led to a slight decrease of cells in which was associated with an increase of cells in G2/M from 21.7 ± 2.9% to 32.7 ± 5.2% (Figure 2B, -TRAIL series). However, when KU5933was combined with TRAIL, the proportion of cells in G2/M decreased from 32.7 ± 5.2% to 23.2 ± 5.1%, indicating that cells in G2/M may be main contributors to the increase in cell death observed in the combination treatment (Figure 2A). The synergistic cytotoxic effect of both AraC or KU5933 was associated with significantly reduced cFLIP expression and enhanced processing of both pro-caspase-8 and pro-caspase-3, and cleavage of cFLIP long (43kDa fragments on the Western blots) (Figure 2C) indicating enhanced DR4/DR5 activation.

Treatment of cells in mitosis sensitises K562 cells to TRAIL

To test whether cells in G2/M are more sensitive to TRAIL, cells were arrested in mitosis using 0.3 μM nocodazole or 0.1 μM colcemid for 16 h and then treated with TRAIL for 24 h. (Supplementary Figure 3). Themicrotubule disrupting drugs arrested up to 80% of cells in mitosis and induced 14.7% and 7.9% cell death, respectively. Combination of nocodazole or colcemid with TRAIL resulted in asynergistic, up to 7-fold increase in apoptosis (Supplementary Figure 3A and 3B).

To further investigate whether cell cycle phase affected TRAIL sensitivity, cells were released from nocodazole-induced mitotic arrest for 6 h to generate cells in G1 phase (Supplementary Figure 3C). Interestingly, treatment with TRAIL led to a 5- and 7-fold increase in apoptotic cells in G1-andM-phase enriched populations, respectively (Figure 2D). It has to be noted that TRAIL-induced apoptosis was higher when the mitotic block was sustained by the continued presence of nocodazole or colcemidin the media (Supplementary Figure 3B and Figure 2D).

In order to test whether the prolonged mitotic arrest or unrelated effects of nocodazole rendered the cells hypersensitive to TRAIL [15], we fractionated asynchronous (AS) cells into fractions of cells in the different phases of the cell cycle by using centrifugal elutriation (Figure 3Aand 3B). Fractions of cells enriched in G1, G1/S, S and G2/M phases were then treated with 50 nM TRAIL for 24 h and induction of cell death measured (Figure 3C). TRAIL sensitivity was higher in the fractions enriched for cells in G1 (24.1 ± 3.3%) and G2/M (23.0 ± 2.0%) than in the G1/S fraction (13.1 ± 5.1%), S phase (12 ± 8%) or S/G2M phase (13.3 ± 8.7%; Figure 3C and Figure 2D).

The expression level of the key components of the TRAIL apoptotic machinery, namely Bcl-2, Bcl-XL, Mcl-1, Bid, XIAP, cFLIP, were also analysed in the elutriation fractions (Figure 3D). As controls of population purity, the level of cyclins E and B1, key regulators of the G1/S and G2/M phases, respectively, were included. In the AS and G1 populations the level of cyclin E is low, consistent with a majority of cells being in the G0/G1 phase, in G1/S population the level of cyclin E peaked before starting to decrease gradually in the populations enriched for cells in S, S/G2 and G2/M. In the AS and the G1 populations the level of cyclin B1 was low, again consistent with the majority of the cells being in G0/G1 phase. The level of cyclin B1 increased in the population enriched for S phase and peaked in the populations enriched in S/G2 and G2/M phase (Figure 3D), confirming the enrichment for the cell cycle phases (Figure 3A and 3B). When looking at proteins of the apoptotic machinery, Bcl-2, Bcl-XL, Mcl-1, Bid, and XIAP showed no cycle-dependent expression. On the contrary, the expression of cFLIP oscillated synchronously with cell cycle progression, with low levels of cFLIP in the populations enriched for cells in G1 and the G2/M phases (Figure 3B), the phases where the cells showed the highest TRAIL sensitivity (Figure 3C).

Discussion

K562 cells are derived from a CML patient with blast crisis showing characteristics of early erythroid cells and expression of the major red cell sialoglycoprotein, glycophorin [8]. Probably through expression of the Bcr-Abl fusion protein, K562 cells are quite drug resistant, and was here used as a model for TRAIL-resistant erythroleukemia [8].

Here we explored the utility of the death ligand cytokine TRAIL as a novel treatment option. We found that araC has a synergistic cytotoxic effect with TRAIL. Similar results have been reported in other cancer types. For example, cisplatin [16], etoposide [16,17] doxorubicin [17], and irradiation [18] have been shown to sensitise leukemia or gliomacells to TRAIL.

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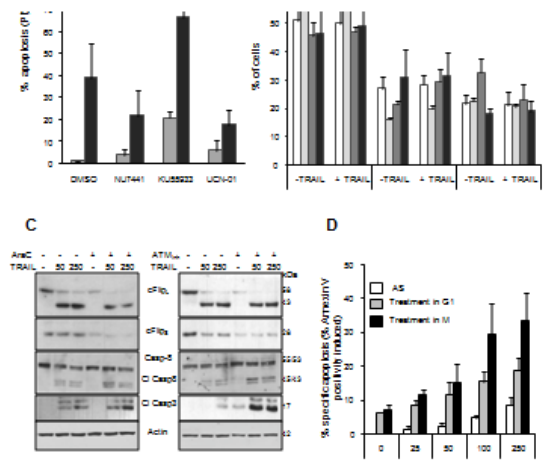

Figure 2: Inhibition of the DDR kinase ATM sensitizes K562 cells to TRAIL-induced apoptosis. K562 cells were treated for 24h with specific inhibitors of DDR kinases [DNA-PK (NU7441, 10 μM) ATM (KU-55933, 10 μM), and Chk1 (UCN-01, 100 nM)] followed by 24h treatment with 250 ng/ml TRAIL, before being harvested for PI staining. Analysis of PI by flow cytometry was used to determine the percentage of cells in Sub-G1 and the percentage of cells in each phase of the cell cycle from the cycling population. Proteins were extracted for western blotting. A) Effect of combination treatment with DDR inhibitors and TRAIL on apoptosis induction determined using PI staining. B) Effect of combination treatment with DDR inhibitors and TRAIL on cell cycle distribution of the cycling population. C) Effect of combination treatment of araC (5 μM) and TRAIL and of ATM inhibitor (KU55933) on the expression of cFLIP, caspase-8, cleaved caspase-8 and cleaved caspase-3. D) Cells in G1 and G2/M are more sensitive to TRAIL-induced apoptosis. K562 cells were mock-treated (AS; white bars) or treated with 0.3 μM nocodazole for 16 h to induce arrest in mitosis. Nocodazole was then removed and M-phase cells were either treated with TRAIL (black bars) or allowed to re-enter the cell cycle for 6 h, and then treated with TRAIL (grey bars, enriched population in G1). The graphs show the mean of three independent experiments ± STD.

The cytotoxic effects of araC are related to its ability to be incorporated into DNA and induce replication fork stalling, which induces S-phase delay and G2/M arrest and triggering the DNA damage response pathway [19-22]. In agreement with another study, where TRAIL has been reported to cause rapid activation of ATM- and DNA-PK-dependent phosphorylation of H2AX and Chk2 [23], we also found that while inhibition of DNA-PK or Chk1 did not influence TRAIL-induced apoptosis, inhibition of ATM also sensitise K562 cells to TRAIL-induced apoptosis.

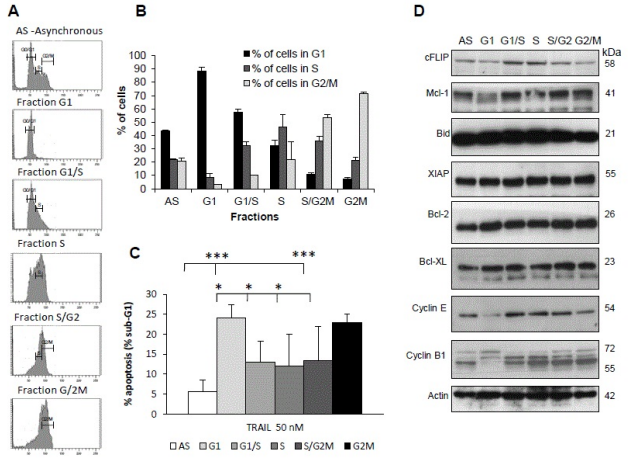

Figure 3: Higher TRAIL sensitivity of cells in G1 and G2/M phase of the cell cycle K562 cells were subjected to centrifugal elutriation to obtain fractions of cells enriched in different phases of the cell cycle before treatment with TRAIL (50 nM). Cells were stained with PI and analysed by flow cytometry, and proteins were extracted for western blotting. A) Representative histograms of K562 cells before (AS) and after centrifugal elutriation. B) Cell cycle distribution of K562 cells before (AS) and after centrifugal elutriation. C) TRAIL sensitivity of an asynchronous population (AS) and fractions of cells enriched in G1, G1/S, S, S/G2M and G2/M treated with 25 ng/ml TRAIL. *p<0.05; ***p<0.001. D) Expression of key components of the apoptotic machinery and of two components of the cell cycle.

The increased TRAIL-sensitivity after ATM inhibition could result from down regulation of c-FLIP as previously reported in melanoma cells [24], and the BH3-only protein Bid has also been shown to be a target of ATM and to control cellular fate in response to DNA damage [25,26]. By connecting death receptor signalling to the mitochondrial amplification loop of the intrinsic pathway [27], Bid could play a role in the sensitisation to TRAIL observed after both araC treatment and ATM inhibition.

We found that cells in the G2/M phase were more sensitive to TRAIL and arresting the cells in G2/M or enriching the culture in mitosis or in G1 using elutriation led to sensitisation to TRAIL. We found that cFLIP has an oscillating expression during the cell cycle with highest expression in G1-S transition and S phases and low expression during G2/M and G1. This is in agreement with the reported JNK-dependent proteasome-mediated decrease in c-FLIP in G2/M [28]. Similarly, the study by Gascoigne found that the apoptotic threshold gradually reduces during prolonged mitosis [29]. The observation that the extent of sensitisation was lower when the mitotic block was removed compared to when the block was sustained supports a role for prolonged mitotic arrest in the increased sensitivity of K562 cells to TRAIL-induced apoptosis [28], rather than the abrogation of the checkpoint [30].

Similar to our results, Ivanov and colleagues observed that ATM inhibition led to G2/M arrest and sensitised cells to TRAIL-induced apoptosis [24]. The study showed that gamma-irradiation-mediated ATM activation led to STAT3-driven cFLIP induction and TRAIL resistance. Inhibition of ATM blocked cFLIP induction and sensitised

melanoma cells to TRAIL, highlighting the multifaceted role of ATM in DNA repair control and our still incomplete understanding of cell fate decision control in response to DNA damage.

Overall, we show that in the G1 or G2/M phases of the cell cycle; erythroid leukemia cells become sensitive to TRAIL-induced apoptosis. This sensitivity can be also achieved and intensified by arresting the cells in G2/M. Further studies would be necessary to determine if the increased sensitivity to TRAIL was due to cFLIP down regulation in G2/M. To translate these findings into clinical use, studies on primary erythroleukemia cells will need to be carried out. Importantly, these results warrant further studies into exploring the efficacy of TRAIL treatment with agents that disturb cell cycle progression, such as cyclin-dependent kinase inhibitors [31].

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