Resistance to TRAIL in mantle cell lymphoma cells is associated with the decreased expression of purine metabolism enzymes

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Abstract. Mantle cell lymphoma (MCL) is a rare aggressive type of B-cell non-Hodgkin's lymphoma. Response to chemotherapy tends to be short and virtually all patients sooner or later relapse. The prognosis of relapsed patients is extremely poor. The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is considered one of the novel experimental molecules with strong antitumor effects. TRAIL triggers extrinsic apoptosis in tumor cells by binding to TRAIL ‘death receptors’ on the cell surface. Recombinant TRAIL has shown promising pro-apoptotic effects in a variety of malignancies including lymphoma. However, as with other drugs, lymphoma cells can develop resistance to TRAIL. Therefore, the aim of this study was to identify the molecular mechanisms responsible for, and associated with TRAIL resistance in MCL cells. If identified, these features may be used as molecular targets for the effective elimination of TRAIL-resistant lymphoma cells. From an established TRAIL-sensitive mantle cell lymphoma cell line (HBL-2) we derived a TRAIL-resistant HBL-2/R subclone. By TRAIL receptor analysis and differential proteomic analysis of HBL-2 and HBL-2/R cells we revealed a marked downregulation of all TRAIL receptors and, among others, the decreased expression of 3 key enzymes of purine nucleotide metabolism, namely purine nucleoside phosphorylase, adenine phosphoribosyltransferase and inosine-5'-monophosphate dehydrogenase 2, in the resistant HBL-2/R cells. The downregulation of the 3 key enzymes of purine metabolism can have profound effects on nucleotide homeostasis in TRAIL-resistant lymphoma cells and can render such cells vulnerable to any further disruption of purine nucleotide metabolism. This pathway represents a ‘weakness’ of the TRAIL-resistant MCL cells and has potential as a therapeutic target for the selective elimination of such cells.

Introduction

Mantle cell lymphoma (MCL) is a rare aggressive type of B-cell non-Hodgkin's lymphoma with an estimated annual incidence in Europe of 0.45/100,000 individuals (1). MCL is a biologically and clinically heterogeneous disease; the immunophenotype of neoplastic cells reflects the phenotype of cells similar to lymphocytes in the mantle zone of normal germinal follicles (2). The genetic hallmark of MCL cells is a translocation between chromosomes 11 and 14, t(11;14)(q13;q32), juxtaposing the gene for immunoglobulin heavy chain and the gene encoding cyclin D1. This results in cyclin D1 overexpression (3,4).

The standard of care for newly diagnosed MCL patients is combined immunochemotherapy alternating rituximab-CHOP (R-CHOP; cyclophosphamide, vincristine, doxorubicin and prednisone) and R-HDAC (high-dose cytarabine). The addition of rituximab and HDAC to CHOP has improved the survival of MCL patients in the last 2 decades from 3 to 5 years. However, the response to therapy tends to be short and virtually all patients sooner or later relapse. There is no standard of care for relapsed or refractory MCL patients. Salvage therapy usually comprises diverse regimens based on fludarabine, gemcitabine, cisplatin, bendamustine, bortezomib (inhibitor of 26S proteasome) or temsirolimus (inhibitor of mTOR). Recently, several new experimental molecules have shown promise in the therapy of relapsed or resistant MCL, including lenalidomide (immunomodulatory agent), ibrutinib (PCI-32765, inhibitor of Bruton's tyrosine-kinase), new monoclonal antibodies (e.g., anti-CD20 ofatumumab), as well as other agents (5). Combination therapies are currently being evaluated in clinical trials; however, novel drugs are required.

The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is considered one of the novel experimental molecules with strong antitumor effects. TRAIL is a type II transmembrane protein from the tumor necrosis factor superfamily (6,7) expressed mostly by cells of the immune system (natural killer cells, cytotoxic T-cells, macrophages and dendritic cells). The main function of this molecule is thought to be in tumor immunosurveillance, but its actual molecular role remains to be elucidated.

TRAIL can trigger extrinsic apoptosis in target cells by binding to TRAIL death receptors located on the cell surface (8). This interaction is performed by a long extracellular C-terminal...
TRAIL receptors in humans (DcR1, DcR2, DR4 and DR5) encoded by separate genes (9,10). However, only DR4 and DR5 contain a functional death domain (structurally conserved protein interaction domain) and are capable of signaling apoptosis. Two decoy receptors (DcR1 and DcR2) lack a functional death domain and inhibit TRAIL signaling by competing with death receptors for TRAIL (9,10). The binding of TRAIL to DR4 or DR5 leads to receptor homotrimerization and formation of the death-inducing signaling complex (DISC) (11). Through the DISC a caspase machinery is activated, which results in apoptosis (12). TRAIL death receptors DR4 and DR5 are ubiquitously expressed, indicating that most tissues and cell types are potential targets of TRAIL signaling (13). Nevertheless, TRAIL seems to induce apoptosis only in tumor cells but not in healthy tissues. Due to its selective pro-apoptotic effect, TRAIL has attracted much attention for its possible use in cancer therapy. In vitro, a recombinant soluble TRAIL molecule has shown cytostatic or cytotoxic effects in a wide variety of tumor cell lines, including leukemia and lymphoma cells, but not in normal cells (6,7,10,11,14-19). The administration of a recombinant soluble TRAIL molecule has been shown to induce the regression or complete remission of tumors in tumor xenograft models (11,20-26). The efficacy of recombinant TRAIL and agonistic antibodies recognizing either receptor DR4 or DR5 has been investigated in numerous clinical trials, as recently reviewed (27).

TRAIL has also shown promising pro-apoptotic effects in a variety of lymphoma cell lines including MCL (15). However, as with other drugs, cancer cells can develop resistance to TRAIL following prolonged exposure to sublethal doses of TRAIL (14,28). Resistance to TRAIL-mediated apoptosis can arise due to changes at the cell membrane level (typically by loss of expression or mutation of functional DR4 and/or DR5 at the cell surface) or on the intracellular level (such as incorrect formation of DISC and aberrant expression of caspases) (29). The successful therapy of malignancies in general, and particularly those with very poor prognosis, such as MCL, depends on the effective management of drug resistance. An in-depth understanding of the processes involved in the development of drug resistance and a detailed description of secondary molecular changes associated with resistance are essential for successful cancer therapy. Specific molecular changes which occur in drug-resistant cells can confer a potential selective disadvantage to such cells and may be used as targets for the effective elimination of drug-resistant lymphoma cells.

The aim of this study was to elucidate the molecular mechanisms responsible for TRAIL resistance in MCL cells, as well as the secondary molecular alterations associated with this process. We also aimed to identify the phenotypic features specific for TRAIL-resistant MCL cells. If identified, these molecular features can be, at least theoretically, used as molecular targets for the effective elimination of TRAIL-resistant lymphoma cells in experimental therapies.

**Materials and methods**

**Cell growth and cellular toxicity assay.** HBL-2 cells were grown in Iscove’s modified Dulbecco's medium in the presence of 10% foetal bovine serum, 1% penicillin-streptomycin solution in a 37°C humidified atmosphere with 5% CO₂. TRAIL-resistant HBL-2/R cells were derived by selective pressure of increasing concentrations of human recombinant TRAIL (Apronex Biotechnologies, Czech Republic) up to 1,000 ng/ml in medium from the wild-type HBL-2 cells in 5 weeks. The toxicity of TRAIL to HBL-2 and HBL-2/R was measured using the colorimetric WST-8-based Quick Cell proliferation Assay kit II (BioVision, San Francisco, CA, USA) according to the manufacturer’s instructions. Briefly, 40,000 cells were seeded in a 96-well plate in 300 μl of medium supplemented with increased concentrations of TRAIL up to 1,000 ng/ml in medium for 1-4 days. After the addition of WST reagent, absorbance was measured on a Sunrise microplate absorbance reader (Tecan Group Ltd., Männedorf, Switzerland) with a 450 nm reading filter and 630 nm reference filter. The absorbance of free medium was used as the background level, triplicate samples were grown and measured for each cell type and TRAIL concentration. Mean values were calculated. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specified otherwise.

**Flow cytometric analysis.** HBL-2 and HBL-2/R cells (2x10⁶ cells for each assay) were washed in PBS buffer (0.5% foetal bovine serum in PBS), stained with phycoerythrin-conjugated antibodies against TRAIL receptors DR4, DR5, DcR1 and DcR2 (anti-hTRAIL R1, anti-hTRAIL R2, anti-hTRAIL R3 and anti-hTRAIL R4; R&D Systems, Minneapolis, MN, USA) and analyzed by flow cytometry in triplicate (FASCant II, BD Biosciences, San Jose, CA, USA). Unstained cells and cells incubated with isotype controls served as the background fluorescence controls.

**Sample preparation for two-dimensional electrophoresis.** HBL-2 and HBL-2/R cells (6x10⁶) were harvested, washed twice with PBS and cell pellets were frozen and stored at -80°C. Samples were thawed and homogenized in lysis buffer [7 M urea, 2 M thiourea, 4% CHAPS, 60 mM dithiothreitol (DTT) and 1% ampholytes (Bio-Lyte 3-10 Buffer, Bio-Rad, Hercules, CA, USA)] and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) for 20 min at room temperature with occasional vortexing. Samples were sedimented at 18,000 x g for 20 min at room temperature, supernatants were collected and protein concentration was determined by the Bradford assay (Bio-Rad). Protein concentrations in all samples were equalized to 3.3 mg/ml by dilution with lysis buffer.

**Two-dimensional electrophoresis.** IPG strips (pH 4.0-7.0, 24 cm; ReadyStrip, Bio-Rad) were rehydrated overnight in 450 μl of sample, representing 1.5 mg of protein. Isoelectric focusing was performed for 70 kVh, with maximum voltage not exceeding 5 kV, current limited to 50 μA per strip and temperature set to 20°C (Protean IEF Cell, Bio-Rad). Six replicates were run for each cell type. Focused strips were briefly rinsed in deionized water, equilibrated and reduced in equilibration buffer supplemented with DTT (6 M urea, 50 mM Tris pH 8.8, 30% glycerol, 2% SDS and 450 mg DTT per 50 ml) for 15 min and then alkylated in equilibration buffer with iodoacetamide (1.125 mg iodoacetamide per 50 ml of the buffer). Equilibrated strips were then secured on 10% SDS-PAGE and secured in place by molten agarose. SDS-PAGE electrophoresis was performed in
a Tris-glycine-SDS system using a 12-gel Protean Dodeca Cell apparatus (Bio-Rad) with buffer circulation and external cooling (20°C). Gels were run at a constant voltage of 45 V per gel for 30 min and then at a constant voltage of 200 V for 6 h. Gels were washed 3 times for 15 min in deionized water to remove redundant SDS. Gels were then stained with colloidal Coomassie Brilliant Blue (SimplyBlue™ Safestain, Invitrogen, Carlsbad, CA, USA) overnight and briefly de-stained in deionized water.

**Gel image analysis and extraction of peptides.** Stained gels were scanned with GS 800 calibrated densitometer (Bio-Rad) and image analysis was performed with Progenesis™ software (Nonlinear Dynamics, Ltd., Newcastle upon Tyne, UK) in semi-manual mode with 6 gel replicates for each cell type. Normalization of gel images was based on total spot density, and integrated spot density values (spot volumes) were then calculated after background subtraction. Average spot volume values (averages from the all 6 gels in the group) for each spot were compared between the groups. Protein spots were considered differentially expressed if their average normalized spot volume difference was >1.5-fold. As determined by the Student's t-test, a p-value <0.05 was considered to indicate a statistically significant difference.

**Protein digestion and peptide extraction.** Spots containing differentially expressed proteins were excised from the gels, cut into small pieces and washed 3 times with 25 mM ammonium bicarbonate in 50% acetonitrile (ACN). The gels were then dried in a SpeedVac Concentrator (Eppendorf, Hamburg, Germany). Sequencing grade modified trypsin (Promega, Madison, WI, USA) (6 ng/µl of trypsin in 25 mM ammonium bicarbonate in 5% ACN) was added. Following overnight incubation at 37°C, the resulting peptides were extracted with 50% ACN.

**Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and identification of selected proteins.** Peptide samples were spotted on a polished steel target plate (Bruker Daltonics, Bremen, Germany) and allowed to dry at room temperature. Matrix solution (3 mg α-cyano-4-hydroxycinnamic acid in 1 ml of 50% ACN containing 0.1% trifluoroacetic acid) was then added. MS was performed on an Autoflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) using a solid nitrogen laser (337 nm) and FlexControl software (Bruker Daltonics) in reflectron mode (Bruker Daltonics) using a solid nitrogen laser (337 nm) and FlexControl software (Bruker Daltonics) in reflectron mode to identify such a cellular 'weakness' of TRAIL-resistant MCL cells. To identify the specific protein expression changes in the TRAIL-resistant cells, we derived a TRAIL-resistant HBL-2 subclone (HBL-2/R) from the originally TRAIL-sensitive HBL-2 cell line, and performed differential analysis of the surface expression of TRAIL receptors and comparative proteomic analysis of the HBL-2/R and HBL-2 cells.

**TRAIL-resistant cell line.** The TRAIL-resistant HBL-2 subclone (HBL-2/R) was derived from the originally TRAIL-sensitive HBL-2 cell line by selective pressure of increasing TRAIL concentration in medium over 5 weeks. While the IC50 for TRAIL in the originally sensitive HBL-2 cells was 1 ng/ml at 48 h (data not shown), the resulting HBL-2/R subclone proliferated in up to 1,000 ng/ml TRAIL concentration in medium and was therefore >1,000-fold more resistant to TRAIL than the HBL-2 cells (Fig. 1).

**TRAIL receptors - flow cytometric analysis of cell surface expression.** The attenuated expression of TRAIL death receptors DR4 and DR5 has been previously described as a cause of TRAIL resistance. We therefore determined the relative expression of TRAIL receptors in HBL-2 and HBL-2/R cells by flow cytometry (Fig. 2). The expression of DR4, DR5, DcR1 and DcR2 in the HBL-2/R cells was markedly decreased compared to the HBL-2 cells. The marked downregulation of death receptors DR4 and DR5 explains the resistance of the HBL-2/R cells to TRAIL, while the downregulation of decoy receptors DcR1 and DcR2 may indicate further, more complex phenotypic changes in the HBL-2/R cells.
In order to identify specific changes in protein expression associated with TRAIL resistance in HBL-2/R cells, we performed comparative proteomic analysis of cellular homogenates of HBL-2/R and TRAIL-sensitive HBL-2 cells. Using two-dimensional electrophoresis of total cell lysates, we reproducibly detected 820 protein spots on Coomassie Brilliant Blue-stained gels. We found 21 protein spots to be significantly quantitatively changed (upregulated or downregulated, change > 1.5-fold; p<0.05) in HBL-2/R cells (Fig. 3). Using MALDI-TOF/TOF mass spectrometry we identified all 21 proteins differentially expressed in HBL-2/R cells (Table I).

Functional annotations of the identified differentially expressed proteins were analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Among the 21 identified proteins we found molecules involved in diverse functions, including cytoskeleton regulation, ribosome synthesis and maturation, RNA metabolism, chromosome translocation, DNA repair and replication, as well as protein folding. However, one pathway was markedly enriched in our set (hsa00230 - purine metabolism) represented by 3 differentially expressed proteins. These 3 molecules are key enzymes of the purine nucleotide metabolism (Fig. 5) and all 3 are downregulated in TRAIL-resistant HBL-2/R cells [PNP (downregulated 1.6-fold in HBL-2/R cells), APRT (downregulated 2.2-fold in HBL-2/R cells) and inosine-5'-monophosphate dehydrogenase 2 (IMPDH2, downregulated 1.6-fold in HBL-2/R cells)].

Verification of proteomic analysis. To confirm the results of proteomic analysis by an independent method we verified the decreased expression of the 2 proteins involved in purine metabolism, namely PNP and APRT, by western blot analysis in HBL-2 and HBL-2/R cell lysates (Fig. 4).

**Discussion**

The downregulation of the 3 key enzymes of purine metabolism can have a profound effect on nucleotide homeostasis in TRAIL-resistant lymphoma cells. Purine nucleotides, the building blocks for synthesis of DNA, RNA and enzyme co-factors, are recruited either from de novo purine synthesis from low molecular weight precursors or by recycling of free nucleobases in the so-called salvage pathway. Both pathways lead to the production of nucleoside-5'-phosphates (Fig. 5). Both pathways can supply cellular demand independently; however, their importance in different tissues is variable. In leukemic and lymphoma cells the salvage pathway is considered the major source of purine nucleotides (30,31).
The de novo synthesis of purine nucleotides requires 5-phosphoribosyl-1-pyrophosphate (PRPP), ATP, glutamine, glycine, CO₂, aspartate and formate to create the first fully formed nucleotide, inosine-5'-monophosphate (IMP). IMP represents a branch point for purine biosynthesis, since it can be converted either to guanosine-5'-monophosphate (GMP) by IMPDH2 (downregulated in HBL-2/R cells) or to adenosine-5'-monophosphate (Fig. 5).

The catabolism of purine nucleotides leads to the liberation of free purine bases by PNP (downregulated in HBL-2/R cells). In the salvage pathway the free bases are reconverted back to nucleoside-5'-monophosphates in a reaction with activated sugar (PRPP) catalyzed by APRT (downregulated in HBL-2/R cells) or hypoxanthine-guanine phosphoribosyltransferase (32) (Fig. 5). Ribonucleotides are converted by ribonucleotide reductase into the corresponding deoxyribonucleotides.

Table I. List of proteins differentially expressed in HBL-2/R cells (difference at least 1.5-fold and statistical significance p<0.05).

<table>
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<th>Spot no.</th>
<th>Swiss-Prot no.</th>
<th>Protein name</th>
<th>Fold change</th>
<th>Mascot score</th>
<th>Sequence cov. (%)</th>
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*aSwiss-Prot no. is the code under which the identified protein is deposited in the Swiss-Prot database. bMascot score helps to estimate the correctness of the individual hit. It is expressed as -10 x log(P) where P is the probability that the observed match is a random event. cSequence coverage is the number of amino acids spanned by the assigned peptides divided by the sequence length.
The delicate balance of enzyme activities and concentrations of products and intermediates are critical for purine (nucleotide) homeostasis. The inhibition of PNP results in the accumulation of its substrate, 2'-deoxyguanosine which is further phosphorylated to deoxyguanosine triphosphate (dGTP). A high intracellular concentration of dGTP inhibits cell proliferation and induces apoptosis (33-35). If APRT is inhibited, accumulated adenine is oxidized to insoluble 2,8-dihydroxyadenine. Accumulation of this precipitate results in cell death (32). Similarly, the inhibition of IMPDH2 leads to depletion of guanosine nucleotides, which blocks DNA synthesis and cell division (36,37).

Disruption of the purine nucleotide metabolism generally results in an accumulation and/or a lack of ribonucleotides or deoxyribonucleotides or metabolic intermediates with potentially cytotoxic consequences. The observed decreased expression of the 3 purine metabolism enzymes affects both de novo synthesis and the salvage pathway of purine metabolism and may also affect purine nucleotide homeostasis in TRAIL-resistant HBL-2/R cells. Such an imbalance may represent a selective disadvantage for the affected cells. Such a 'weakness' may not be apparent under normal circumstances but may become critical under stress or unfavorable conditions. As the proliferation rates of HBL-2/R and HBL-2 cells are comparable, the proposed imbalance in purine nucleotide metabolism in TRAIL-resistant cells is possibly mild and/or well compensated in vitro. However, this 'weakness' may become apparent due to lack of building blocks for DNA and RNA synthesis in the environment or upon further disruption of purine metabolism. Since both pathways of purine metabolism are compromised in TRAIL-resistant MCL cells, these cells should be vulnerable to further inactivation of purine nucleotide metabolism enzymes. Therefore, drugs that target (already disbalanced) purine metabolism should be highly cytotoxic to TRAIL-resistant cells (compared to non-malignant cells) and may therefore be selectively effective in the elimination of TRAIL-resistant MCL cells in experimental therapy. There are several approved inhibitors of purine metabolism, such as methotrexate (inhibits purine de novo synthesis via dihydrofolate reductase) (38), ribavirin and mycophenolic acid (inhibitors of IMPDH2) (39,40) or forodesine (a novel inhibitor of PNP) (41,42), available for clinical use.

The adaptation of cancer cells to cytostatic and cytotoxic drugs is associated to a certain degree with extensive changes in the cell phenotype. Some of the molecular changes, although seemingly unrelated to the mechanism of resistance, can provide a selective disadvantage to the cells and such a 'weakness' may be used as a potential therapeutic target. By the presented proteomic analysis of the changes associated with resistance to TRAIL in MCL HBL-2 cells, we demonstrated the downregulation of all types of TRAIL receptors and identified the altered expression of several proteins including 3 enzymes of the purine metabolism pathway. This downregulated pathway potentially represents a 'weakness' of the TRAIL-resistant MCL cells and has potential as a therapeutic target for the selective elimination of such cells in the future.
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