Apoptotic effect of recombinant and stem cell-produced TRAIL protein on rhabdomyosarcoma cells.

Ph.D. thesis

Helga Barti-Juhász

Semmelweis University

Science of Pathology, School of Ph.D. Studies

Advisor: István Peták, M.D., Ph.D., Semmelweis Univ.

Examiners: Zsuzsa Szondy, M.D., D.Sc., Habil., Univ. Debrecen
Mónika Csóka, M.D., Ph.D., Semmelweis Univ.

Comprehensive exam committee:
Chair: György Fekete M.D., D.Sc., Habil.,Semmelweis Univ.
Members: Janina Kulka, M.D., Ph.D., Habil Semmelweis Univ.
Zsolt Orosz, M.D., Ph.D., Habil, Nat'l. Inst of Oncol.

Budapest, 2012.

I. INTRODUCTION

Rhabdomyosarcoma (RMS) is the most frequent juvenal cancer originating from skeletal muscle, and patient survival is poor in the case of metastatic disease. New targeted therapeutics are critically needed My mentor, István Peták and his coworkers have shown that DR5 receptor of TRAIL was expressed on the 7 RMS cell lines they examined and around half of the lines were highly sensitive for the oligomerized hr-TRAIL-induced caspase-mediated apoptosis.. They have also shown that caspase-8 expression of RMS cells correlated well with sensitivity to TRAIL.

TRAIL-related therapeutic products in various phases of clinical trials are in the pipeline of several biotechnological and pharmaceutical companies. The recombinant human TRAIL (dulanermin) was in phase I as a single agent but without a major breakthrough. From the 71 treated patents, two patients with chondrosarcoma responded partially and two other patients had serious side effect provoked by tumor lysis syndrome. Dulanermin in combination with chemotherapeutics is in phase II. Agonistic antibodies for DR4 and DR5 TRAIL-receptors are also in phase II. Based on preclinical data, RMS were included the phase I/II clinical trial of an anti-DR5 mab (drozitumab) targeted against childhood sarcomas.

For enhancement of TRAIL and TRAIL-R antagonist mabs anticancer activity we need to know the molecular composition of the signal pathways initiated by the TRAIL-Rs. Resistance against TRAIL-related therapy can be overcome by correct diagnostic selection of sensitive patients and/or application of an appropriate combination agent.

TRAIL is a type II transmembrane protein that can be solubilized by a cystein protease in the membrane (114-281 amino acid) and its active form is a homotrimer. TRAIL seems to be the primary death ligand applicable in

cancer therapy because around half of the cell lines from any origin are sensitive for the apoptosis induced by TRAIL but not their normal counterparts, and has no obvious toxic effect. TRAIL has two apoptotic death receptor containing full death domains in the cytoplasm (DR4 and DR5) and three decoy receptors of which two are membrane anchored (DcR1 and DcR2) and one soluble (OPG). After engagement of the trimerized TRAIL with its DR4/DR5 receptors, FADD, caspase-8,-10, and/or c-FLIP proteins are recruited to the DISC (death initiation signal complex). In cells with type I apoptosis signal, initiator caspases (-8 or -10) can directly activate the executioner caspases (-3, -6, -7) by defeating XIAP anti-caspase activity. In cells with type II apoptosis signal, initiator caspases are not able to break through the shield of strong XIAP defend and amplifier signal from mitochondria is required for full blown apoptosis. At the mitochondria the Bcl-2 family proteins regulate the apoptotic signal. Over-expression of Bcl-2 or Bcl-X_L can block the mitochondrial apoptotic signal.

The TRAIL-sensitizing effect of proteasome inhibitors (PSI) for apoptosis is well known in epithel cell lines. Inhibition of proteasome core protease activity with bortezomib, the first clinically applicable PSI, changes the level of various molecular components for enhancing the apoptotic pathway. Bortezomib up-regulates the expression of DR4 and DR5, promotes the assembly of the DISC, decreases expression of c-FLIP and prevents of degradation of active caspases-8 at death receptor proximity. In type II apoptosis signal bortezomib up-regulates Bim or Bik expression, prevents degradation of Bax and tBid and decreases the level of Bcl-2 and Bcl-X_L. Furthermore, bortezomib inhibits the pro-survival NF-κB activation and consequently XIAP expression promoting type I apoptotic signaling. Sparse results obtained with combination treatment in sarcoma cell came from Ewing's sarcoma cell lines but not with rhabdomyosarcoma cells.

The membrane bound and soluble TRAIL was shown to induce diverse signal pathways. The cross-linking of DR5 receptors (but not DR4) was a requirement for effective apoptosis induction in tumor cells. Therefore DR5 are more sensitive for membrane bound than soluble TRAIL. As DR5 is the primarily expressed death receptor in rhabdomyosarcoma cell lines, it was proposed that membrane bound TRAIL expressed by cells may enhance the anticancer effect of TRAIL if these cells can be delivered to tumors. Mesenchymal stem cells (MSC) could be an appropriate choice for carrying TRAIL as MSCs were shown homing favorably into tumors while having low immunogenic reactivity even in allo- or sometimes in xeno-recipients.

II. AIMS

1. We would like to enhance the apoptotic effect of the soluble r.h. TRAIL[114-281] on rhabdomyosarcoma cell lines by revealing possible mechanisms of resistance and applying combination treatments to overcome the apoptotic resistance.
   a.) We have noticed that the Bcl-2 protein was overrepresented in the RD cells resistant relatively to the TRAIL–induced apoptosis. We asked if Bcl-2 up-regulation does really contribute to the TRAIL-resistance in RD cells.
   b.) Whether inhibition of proteasome activity could overcome the apoptosis resistance mediated by Bcl-2 up-regulation?
   c.) Whether is it possible enhancing the TRAIL sensitivity of other rhabdomyosarcoma cell lines possessing defects at the proximity of the death receptors?

2. Investigation of the apoptotic effect in cancer cells induced by TRAIL expressed in MSC cells.
   a.) Design and production of DNA vectors for expression of full, membrane localized TRAIL or secretable TRAIL possessing an isoleucine zipper trimerization domain.
   b.) Application of nucleoporation for transfection of the DNA vectors into MSC cells.
   c.) Apoptotic effect of TRAIL expression in MSC cells and in the co-cultured tumor cells (using RD sarcoma and HeLa carcinoma cell lines).

III. MATERIALS AND METHODS

III. 1. Cell cultures

Az RD, RD-GFP, RD-Bcl2 and Rh41 rhabdomyosarcoma cell lines were cultured in RPMI 1640 + 10% FBS media, HeLa carcinoma cells were grown in DMEM + 10 % FBS + 1% pen/strtep media. Cells were preincubated in 24 well plates with LLnL (1μM) and bortezomib (Bzb) (1μM, 30 nM) and were co-exposed to h.r. TRAIL (25 ng/ml, 24 hrs).

III. 2. Construction of DNA vectors containing TRAIL sequences

For the ILZ-TRAIL gene construct we optimalized the 223 bp sequence for PCR and "fragmented" it for short oligo-sequences that were synthesized by a vendor. Dual assimetric, overla extension and full length product amplification PCR were used for building up the hole 223 bp sequence. This synthetic gene product was conjugated to the sequence of extracellular TRAIL domain (525 bp) and cloned into an expression DNA vector. We produced a another DNA vector containg the full length of TRAIL. The TRAIL sequence was originated from pORF-hTRAIL vector. The DNA constructs were cloned into pCR-Blunt II-TOPO and pcDNA3.1/V5His-TOPO vectors. Sequencing was performed with ABI Prism 310 genetic analyser.

III. 3. Isolation, culture and nuclefection of MSC cells

Mesenchymal stem cells (MSCs) were derived from bone marrow and adipose tissues. Mononuclear cells were separated with density gradient centrifugation. MSCs were cultured as adherent fraction in DMEM (low glucose) + 10 % FBS. MSC cells were transduced by a method combining chemical transfection and electroporation and called nucleofection (AMAXA-nukleofektor). The effectiveness of nucleofection was monitored

with pmax-GFP DNA-vector detecting GFP fluorescence by confocal microscope or flow cytometry.

III. 4. Co-culture of tumor and MSC cells

Co-culture of cells were performed as direct-mix of cells (allowing cel-cell contact) and in trans-well plates (allowing only paracrine, soluble fraction contact).

BM-MSC cells were nucleofected with the full length TRAIL containing vector (or a control vector) and directly mixed with RD cells in 6 well plates and cultured in 1:1 mix of their media. Cell number were counted after 5 days co-culture in a Bürker chamber. In the trans-well plates (Corning transwell, 0,4 µm) BM-MSC nucleofected with ILZ-TRAIL or full TRAIL containing DNA-vectors were co-cultured with RD cells and after 24 hrs subG1 apoptotic cells were detected by flow cytometry.

AD-MSC-TRAIL cells were co-cultured with HeLa cells in direct-mix or trans-well cultures at various target-effector ratio (Corning transwell, 0,4 µm) 1:1, 1:2 és 1:5, T:E. Dead cells were counted after propidium jodide staining by flow cytometry.

III. 5. Flow cytometric detection of apoptotic cell with DNA fragmentation

Apoptosis usually accompanied with nucleosomal DNA fragmentation. If cell were fixed in ethanol, fragmented DNA could be extracted by a weak alkaline solution allowing detection of apoptotic cells as weakly stained with PI nucleic acid stain and consequently weakly fluorescenct events by flow cytometry (FACSCalibur, Becton Dickinson).

III.6. Immunephenotyping

The immune phenotype of AD-MSC cells were characterized by our collaboration partner with the following monoclonal abys: PerCP-anti-

CD45, APC-anti-CD14, PE-anti-CD34, PE-anti-CD90, PE-anti-CD73, PE-anti-CD105. After transfection, AD-MSC-TRAIL, AD-MSC-GFP, AD-MSC cells were stained with PE-anti-TRAIL, PE-anti-TRAIL-R1, PE-anti-TRAIL-R2 and isotype control abys.

III. 7. Western blot

Treated cell samples were lysed and protein were separated on a polyacrylamide gel, blotted to PVDF membrane and labeled with anti-Bcl-2, anti-hTRAIL or anti-β-actin primary abys. Vectastain ABC-kit and ECL were used to visualize protein bands. Intensity of the blots were detected by Eagle Eye gel-doc system and quantified with ImageJ software.

III. 8. ELISA

MSC cells nucleofedted with ILZ-TRAIL or full-TRAIL DNA-vectors were cultured for 4 days and TRAIL content of the supernatants of the cultures were detected with TRAIL/TNFSF10 ELISA DuoSet kit everyday from fresh media and after 4 accumulation.

III. 9. Statistics

Combination treatment efficacy were quantified with a simple synergy factor (SF) calculation. For statistical analysis two tailed Student t-test were applied (Microsoft-Excel) homocedastic comparison of samples with similar deviations and non-homocedastic comparision of samples with dissimilar deviations. $p<0,05$ was considered as statistically significant.

IV. RESULTS

IV. 1. TRAIL and proteasome inhibitors induced apoptosis synergisticaly in rhabdomyosarcoma cells

RD cells were exposed to h.r. TRAIL[114-281] for 24 hrs. The 50% effective concentration of TRAIL for induction of apoptosis was EC_{50} ≈25 ng/mL, and most of the RD cells (~90%) died above of 200 ng/mL concentration of TRAIL. We observed that Bcl-2 expression doubled in RD cells survived the treatment with 25 ng/mL of TRAIL. Various epithel cell types have become more sensitive for TRAIL-induced apoptosis after inhibition of their proteasome core protease activity. Therefore we exposed the RD cells for PSIs (LLnL, bortezomib) that significantly enhanced the ratio of apoptotic cells after TRAIL treatment in combination. Also, PSIs suppressed the upregulation of Bcl-2 expression in the surviving RD cells.

For studying the role of Bcl-2 protein in TRAIL-resistance we created an RD cell line constitutively expressing Bcl-2 and/or GFP (green fluorescence protein) after transduction with retroviral vectors (RD-Bcl-2, RD-GFP). RD-Bcl-2 cells become almost completely resistant to TRAIL-induced apoptosis that was overcome by treatment with PSIs also. Combination treatment provided high value for synergy factor in case of both LLnL (SF_{LLnL}=2.4) and bortezomib ($SF_{Bzb1\mu M}$=14.9 és $SF_{Bzb\ 30nM}$=4.6).

Mechanistic studies using general caspase inhibitor zVD.fmk revealed that the combination treatment resulted in caspase-dependent apoptotic cell death in all RD cell lines.

We also shown that another TRAIL-resistant RMS cell line Rh41 was also synergistically sensitized for TRAIL-induced apoptosis with bortezomib.

IV. 2. Production of DNA vectors containing TRAIL sequences and functional comparision of them in MSC cells

We constructed two DNA vector (plasmid) that contained different TRAIL sequences. One with full length TRAIL[1-281] and another with the extracellular domain of TRAIL[114-281] associated with a sequence designed by us and contained the secretion signal sequence of the human growth hormone and an isoleucine zipper (ILZ) domain appropriate for trimerization of TRAIL and these two were connected with a consensus sequence for furin protease cleavage site allowing separation of the ILZ-TRAIL protein sequence from the secretion signal peptide . MSC cells were nucleofected with these DNA-vectors and the ratio of transfected cells were relatively high (52±17%) detected by a DNA vector contained the GFP indicator.

The amount of TRAIL was detected with ELISA method in the supernatant of the nuclefected MSC cells. Full-TRAIL construct produced daily 6-8 ng/mL TRAIL while ILZ-TRAIL[114-281] construct generated maximally 112 pg/mL TRAIL after 4 days. The paracrine apoptotic effect of the solubilized TRAIL was examined on RD rhabdomysarcoma cells in trans-well experiments. Neither the ILZ-TRAIL nor the full-TRAIL expressing MSC cells could influence the cell death in the separated RD cells indicating that TRAIL production were not strong enough for paracrine induction of apoptosis in RD cells.

IV. 3. Inhibition of growth of tumor cell number by TRAIL-expressing MSC cells derived from bone marrow and adipose tissue

Bone marrow derived MSC (BM-MSC) were exposed to r.h. TRAIL for 24 hrs but we could not detect the sign of apoptosis.. We showed the full length TRAIL expression by Western blot after nucleofection of MSC cells with the full-TRAIL containing DNA-vector. Allowing close proximity of MSC and RD RMS cells we detected strong inhibition of growing cell number of RD cells cultured together with BM-MSC-TRAIL cells (84 % inhibition) but not with BM-MSC-GFP cells at moderate target-effector cell ratio (T:E=4:1).

In case of adipose tissue derived MSC cells (AD-MSC), retroviral transduction resulted in full-TRAIL expression that was detected by immunefluorescence staining of MSC cells with anti-TRAIL mab and by the presence of TRAIL in the supernatant with ELISA method. AD-MSC-TRAIL cells provided 62% of apoptosis in the TRAIL sensitive HeLa carcinoma cells in cultures allowing close contact of the target and effector cells (T:E=1:5). Apoptosis of HeLa cells were effectively reduced by an antagonistic anti-TRAIL mab. In trans-well experiments the isolated AD-MSC-TRAIL cells also induced apoptosis in significant number of HeLa cells, but the apoptosis ratio was less than one-third of apoptosis detected at direct contact of target and effector cells (T:E=1:5).

AD-MSC cells expressed TRAIL-R2 (DR5) but not TRAIL-R1 (DR4). DR5 expression was upregulated by the proteasome inhibitor bortezomib (50 nM). Bortezomib induced apoptosis in a moderate but significant number of AD-MSC-TRAIL cells but not in AD-MSC-GFP cells indicating that PSI sensitize the MSC cells for TRAIL-induced apoptosis.

V. CONCLUSIONS

1. We can conclude the following about the soluble and recombinant TRAIL[114-281] (dulanermin) effect on RMS cell lines:

a.) The upregulated and constitutive expression of Bcl-2 contributed to the apoptosis resistance of the surviving RD cells exposed to TRAIL. This implicate that RD cells are type II cells that require the induction of the mitochondrial signal pathway to complete the death receptor initiated apoptosis.

b.) The proteasome inhibitors overcome the TRAIL-resistance mediated by Bcl-2. We propose that the apoptotic pathway in RD cells exposed to the combined treatment change from type II to type I.

2. We can conclude the following about the apoptotic effect of TRAIL vectors expressed in mesenchymal stem cells (MSC):

a.) Both the bone marrow and adipose tissue derived, TRAIL expressing MSC inhibited the growth of cell number in tumor cell cultures.

b.) Both nucleoporation and retroviral gene transfer were appropriate methods to achive functional TRAIL expression in MSC cells.

c.) The close contact of TRAIL expressing MSC and targeted tumor cells significantly enhance the apoptosis in target cells.

d.) In presence of proteasome inhibitors even MSC cells become more sensitive to TRAIL-induced apoptotsis either as a bystander effect of the self produced TRAIL or as externally added r.h. TRAIL. For this reason the anti-tumor cell effect of the combination of TRAIL and a PSI requires further experimental confirmation.

VI. LIST OF PERSONAL PUBLICATIONS

Scientific publications closely related to the theme of the dissertation

1. **Barti-Juhasz H**, Mihalik R, Nagy K, Grisendi G, Dominici M, Petak I. Bone marrow derived mesenchymal stem/stromal cells transduced with full length human TRAIL repress the growth of rhabdomyosarcoma cells in vitro. Haematologica. 2011 Mar;96(3):e21-2.

2. Grisendi G, Bussolari R, Cafarelli L, Petak I, Rasini V, Veronesi E, De Santis G, Spano C, Tagliazzucchi M, **Barti-Juhasz H**, Scarabelli L, Bambi F, Frassoldati A, Rossi G, Casali C, Morandi U, Horwitz EM, Paolucci P, Conte P, Dominici M. Adipose-derived mesenchymal stem cells as stable source of tumor necrosis factor-related apoptosis-inducing ligand delivery for cancer therapy. Cancer Res. 2010 May 1;70(9):3718-29. Epub 2010 Apr 13.

Other scientific publications

1. Nagy K, Székely-Szüts K, Izeradjene K, Douglas L, Tillman M, **Barti-Juhász H**, Dominici M, Spano C, Luca Cervo G, Conte P, Houghton JA, Mihalik R, Kopper L, Peták I. Proteasome inhibitors sensitize colon carcinoma cells to TRAIL-induced apoptosis via enhanced release of Smac/DIABLO from the mitochondria. Pathol Oncol Res. 2006;12(3):133-42. Epub 2006 Sep 23.

2. Pintér Ferenc, Várkondi Edit, **Barti-Juhász Helga**, Árvai Kristóf, Őrfi Zoltán, Peták István. Az epidermális növekedési faktor receptor

jelátviteli útjai és molekuláris biológiája. The signal transduction and molecular biology of epidermal growth factor. Orvosképzés 2009 LXXXIV éf. 3:163-170

3. **Helga Barti-Juhász**, András Pazsitka, Istvan Petak, Rudolf Mihalik. Altered Expression of a Broad Range of Protease Genes in SH-SY5Y Neuroblastoma Cells after Bortezomib Exposure Biochemica, 2009; 4: 24-26.

4. Balázs Jóri, **Helga Barti-Juhász**, András Pazsitka, Matan Siterman, István Peták. Expression Analysis of ABC Transporters in Lung Cancer Cell Lines Using a RealTime ready qPCR Focus Panel Biochemica, 2009; 4: 27-28.

5. **Helga Barti-Juhász**, András Pazsitka, Balázs Jóri, Kristóf Árvai, Dániel Erös, György Kéri, István Peták1, Rudolf Mihalik. Altered Expression of a Broad Range of Protease Genes in SH-SY5Y Neuroblastoma Cells after Bortezomib Treatment. Cancer res application note 2010

6. Arvai K, Nagy K, **Barti-Juhász H**, Peták I, Krenács T, Micsik T, Végső G, Perner F, Szende B. Molecular Profiling of Parathyroid Hyperplasia, Adenoma and Carcinoma. Pathol Oncol Res. 2011 Dec 24. [Epub ahead of print]