ORIGINAL ARTICLE

IONIZING RADIATION SENSITIZES LEUKEMIC MOLT-4 CELLS TO TRAIL-INDUCED APOPTOSIS

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Summary: One of perspective approaches in treatment of hematological malignancies is activation of death receptors for TRAIL. However, leukemia cells studied to date have shown variable susceptibility to TRAIL. Our study demonstrates that cells of acute T-lymphoblastic leukemia MOLT-4 are resistant to TRAIL and that ionizing radiation in the therapeutically achievable dose of 1 Gy sensitizes TRAIL-resistant cells MOLT-4 to the TRAIL-induced apoptosis by increase in death receptors for TRAIL DR5. When TRAIL is applied after the irradiation in the time of increased DR5 positivity more efficient cell killing is achieved.

Key words: *TRAIL; Leukemia; Ionizing radiation; DR5; Apoptosis*

Introduction

Therapeutic strategies that target tumor cells without compromising normal tissue function are being intensively sought. One such perspective approach in treatment of hematological malignancies is activation of so called death receptors. The family of death receptors include Fas (CD95), tumor necrosis factor receptor (TNFR), death receptor 3 (DR3/wsl-1), death receptor 4 (DR4, TRAIL-R1) (11) and death receptor 5 (DR5, TRAIL-R2) (15). Of these the most promising are DR4 and DR5, the receptors for tumor necrosis factor–related apoptosis inducing ligand – TRAIL. By binding to these receptors, TRAIL, or its agonists, activate extrinsic apoptotic pathway. They induce trimerization of the receptor and recruitment of Fas-associated death domain adaptor protein (FADD). FADD in turn recruits caspase 8 and 10. This complex is called DISC – death inducing signaling complex. Within this complex caspase 8/10 is activated, which triggers apoptosis (1, 2, 10).

An alternative pathway for apoptosis onset is the intrinsic pathway (12). The intrinsic apoptotic pathway is triggered in response to DNA damage, of which the most severe are double-strand breaks of DNA (DSB). Recognition of DSB causes an increase in and activation of p53 and affects the balance of proapoptotic and antiapoptotic members of the Bcl-2 family, resulting in release of cytochrome c from mitochondria and activation of caspase 9. A typical factor which induces apoptosis by activation of intrinsic pathway is ionizing radiation (IR).

However, these pathways do not work independently. Induction of apoptosis through the extrinsic pathway can be controlled by regulation of the amount of proteins required for DISC formation, mainly DR4 and DR5. Through the activation of various transcription factors, the expression of DR4 and DR5 can increase followed by the induction of apoptosis (7). Treatment of cancer cells with glucocorticoids and interferon gamma can increase DR5 expression in cells with mutated p53 (9). Many transcription factors seem to be involved in transcriptional control of TRAIL receptors, e.g. NFκB, p53, STAT1 (5). TRAIL has been found to kill cancer cells selectively, with low toxicity to normal cells. TRAIL induces apoptosis in many haematological malignancies (i.e. acute lymphatic, myeloid and promyelocytic leukemia). Unfortunately, in vitro resistance to TRAIL-induced apoptosis in hematological malignancies has been observed and TRAIL may thus have only a limited role as a single agent for these cancers (18).

It has been shown previously that antileukemic drugs such as etoposide and Ara-C can induce DR5 increase in leukemia cells and sensitize the cells to TRAIL effects (3, 16, 17). Also, IR caused significantly enhanced positivity of DR5 receptor 24 h after higher dose irradiation (6 and 8 Gy) of TRAIL-sensitive cells of promyelocytic leukemia HL-60. In these cells preincubation with TRAIL also led to an increase in proapoptotic t-Bid and decrease of antiapoptotic Mcl-1 and increased IR-induced apoptosis (13).

Now we are studying the effect of IR, a known activator of the intrinsic apoptotic pathway, on the amount of DR5 receptor in T-lymphocyte leukemic cells MOLT-4 and we are exploring the effect of the combination of TRAIL and IR on cell death and apoptosis induction.

Materials and Methods

Cell culture and culture conditions

MOLT-4 cells were obtained from American Type Culture Collection (University Blvd., Manassas, USA). The cells were cultured in Iscove's modified Dulbecco's medium (Sigma) supplemented with 20 % fetal calf serum, 0.05 % L-glutamine, 150 UI/ml penicillin, 50 μg/ml streptomycin in humidified incubator at 37 °C and controlled 5 % CO_2 atmosphere. The cultures were divided every 2nd day by dilution to a concentration of 2 x 10^5 cells/ml. Cell counts were performed with a hemocytometer. Cell membrane integrity was determined using the Trypan Blue exclusion technique. Cells in the maximal range of 20 passages were used for this study.

Gamma irradiation

Aliquots of 10 ml of cell suspension ($2x10^5$/ml) were plated into 25 cm^2 flasks (Nunc) and irradiated at room temperature using ^{60}Co γ-ray source with a dose-rate of 0.5 Gy/min at a distance of 1 m from the source. After the irradiation the flasks were placed in a 37 °C incubator with 5 % CO_2 and the aliquots of the cells were removed at various times after irradiation for analysis. The cells were counted and cell viability was determined with the Trypan blue exclusion assay.

TRAIL (TNF-related apoptosis-inducing ligand)

For the experiments, Killer TRAIL™ (Alexis, Germany) was used. TRAIL was diluted in HEPES buffer pH 7.7, containing 300 mM NaCl, 0.006 % Tween 20, 1 % sucrose, 0.5 mM DTT (dithiotreitol).

Cell cycle analysis

The cells were collected and washed with a cold PBS (phosphate buffered saline) and fixed with 70 % ethanol. For the fixation of low molecular fragments of DNA the cells were incubated for 5 min at room temperature in a phosphate buffer and then stained with a propidium iodide (PI) in the Vindelov's solution for 30 minutes at a temperature of 37 °C. The fluorescence (DNA content) was measured with the Coulter Electronic (Hialeah, FL, USA) apparatus. A minimum of 10,000 cells analyzed in each sample has served to determine the percentages of cells in each phase of the cell cycle using the Multicycle software.

DR5 and CD7 antibody, apoptosis detection

For apoptosis detection we used Apoptest-FITC kit (DakoCytomation, Brno, Czech Republic). During apoptosis, cells expose phosphatidylserine at the cell surface. Annexin V is a phospholipid binding protein which, in the presence of calcium ions, binds selectively and with high affinity to phosphatidylserine. For detection of CD7 surface marker we used PE-conjugated anti-human CD7-PE (8H8.1, IgG2b-IM1429) obtained from Immunotech (Marseille, France). For detection of TRAIL receptor DR5, mAb

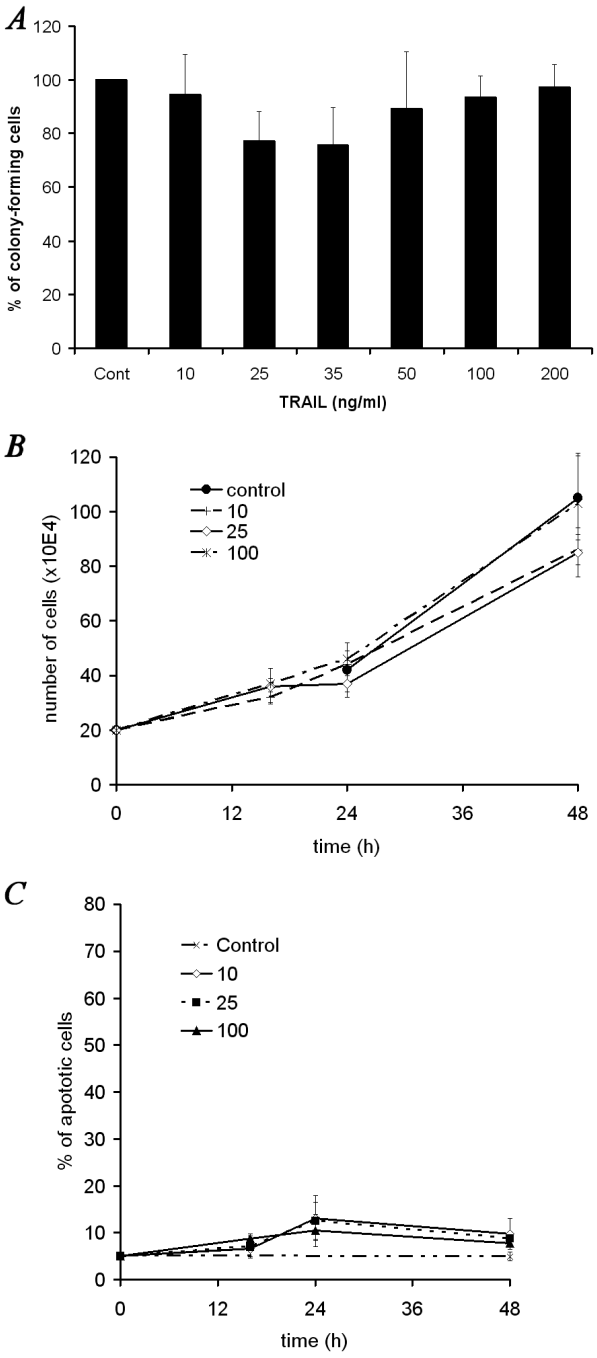

Fig. 1: Effect of TRAIL on MOLT-4 cells.
A: Colony-forming assay. The colonies (containing 40 or more cells) were counted after 14 days of incubation in 5 % CO_2 at 37 °C.
B: Proliferation of the cells in medium containing 10, 25 or 100 ng/ml TRAIL
C: Apoptosis induction by 0, 25 or 100 ng/ml TRAIL determined by flow-cytometric analysis during 48 h of exposure. The data represent mean values from three experiments ± standard deviation. TRAIL did not have significant effect (p>0,1 by Students' T-test).

to DR5 clone HS201 (obtained from Alexis, Germany) was used. The cells were incubated with mAb for 15 min at room temperature. Then the cells were washed with PBS containing 5 % FCS and 0.02 % NaN_3 and incubated with anti-mouse IgG(Fcγ)-PE (obtained form Beckman-Coulter, USA) for 15 min at room temperature. Flow cytometric analysis was performed on a Coulter Epics XL flow cytometer equipped with a 15 mW argon-ion laser with excitation capabilities at 488 nm (Coulter Electronic, Hialeah, FL, USA). A minimum of 10 000 cells was collected for each sample in a list mode file format.

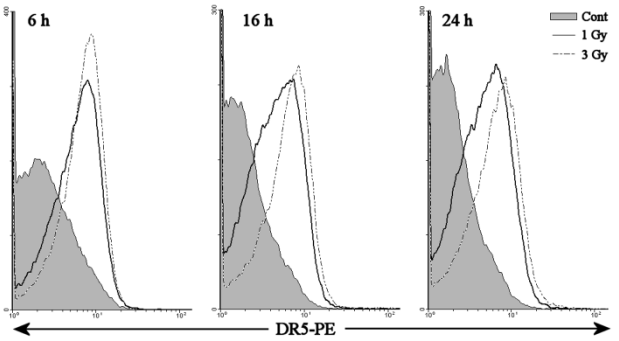

Fig. 2: Increase of DR-5 receptors after irradiation.
MOLT-4 cells were exposed to 1 or 3 Gy of gamma radiation and changes in amount of DR5 were evaluated by flow-cytometry 6, 16 and 24 h after the irradiation.

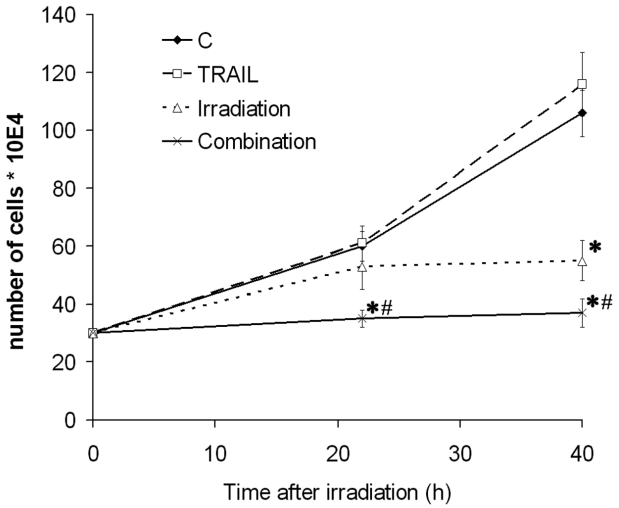

Fig. 3: Combined effects of irradiation and TRAIL on cell proliferation.
MOLT-4 cells were irradiated by the dose of 1 Gy and TRAIL in final concentration 100 ng/ml was added to the culture medium 16 h after the irradiation. The data represent mean values from three experiments ± standard deviation.
*: significantly different to control; #: significantly different to irradiation alone ($p<0,01$ by Students' T-test).

Statistical analysis

The results were statistically evaluated with Student's t-test. The values represent mean ± SD (standard deviation of the mean) from three independent experiments. Statistical significance of the difference of means in comparable sets is indicated in figures.

Results

MOLT-4 cells are resistant to TRAIL

TRAIL itself has no significant long-term effect on MOLT-4 cells. Incubation with TRAIL did not significantly reduced proliferation rate of MOLT-4 cells (Fig. 1B), did not significantly reduce the number of cells forming colonies (Fig. 1A) and did not induce apoptosis (Fig. 1C). Using flow cytometric detection, only 7 % of control MOLT-4 cells are positive for DR5 receptor.

Irradiation causes increase of DR5 receptors

We evaluated changes in expression of TRAIL DR5 receptors after exposure of MOLT-4 cells to different doses of ionizing radiation. As shown in Fig. 2, ionizing radiation induced a significant increase of DR5 receptors after irradiation with doses of 1 and 3 Gy, as detected by flow cytometry 6 to 24 hours after irradiation. The increase in DR5 was already apparent 6 h after the irradiation (C 7.1 %, 1 Gy 35.8 %, 3 Gy 45.7 %), rose further at 16 h (C 6.3 %, 1 Gy 40.7 %, 3 Gy 59.8 %) and the DR5 positivity persisted also at 24 h (C 6.9 %, 1 Gy 44.1 %, 3 Gy 60.4 %).

Irradiation sensitizes MOLT-4 cells to TRAIL-induced apoptosis

As the difference between DR5 positivity 16 h and 24 h after irradiation was not considerable, we decided to add TRAIL to the culture medium 16 h after the irradiation. The cells were irradiated with the dose of 1 Gy and 16 hours later TRAIL was added to culture medium in a final concentration of 100 μg/l. Combination of these two factors caused significantly more intensive proliferation arrest (Fig. 3) and induced apoptosis in a higher percentage of cells in the comparison to only irradiated cells, as detected by Annexin V binding (IR 41 %, IR+TRAIL 52 %; 40 h after the irradiation – Fig 4A) and DNA content analysis (IR 22 %, IR+TRAIL 33 %; 40 h after the irradiation – Fig. 4B). The cells were losing CD7 positivity during apoptosis. Fig. 5 shows the changes of CD7 positivity and Annexin V binding 22 h after the irradiation.

Discussion

The therapy of human leukemia has experienced great advances during recent years. However, as for leukemias of lymphocytic origin, chronic lymphocytic leukemia is considered a resistant disease that desperately needs new therapeutical strategies and adults with acute lymphoblastic leukemia almost universally relapse after conventional chemotherapy (7).

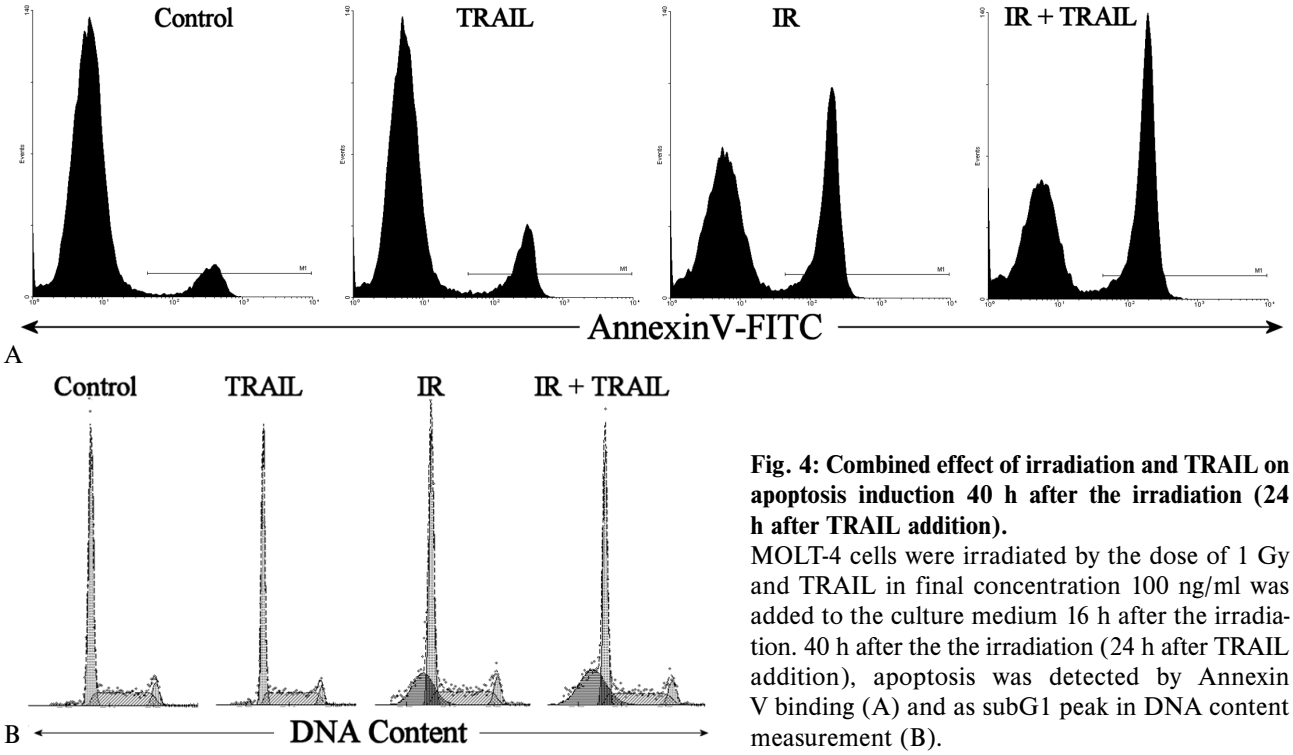

Fig. 4: Combined effect of irradiation and TRAIL on apoptosis induction 40 h after the irradiation (24 h after TRAIL addition).
MOLT-4 cells were irradiated by the dose of 1 Gy and TRAIL in final concentration 100 ng/ml was added to the culture medium 16 h after the irradiation. 40 h after the the irradiation (24 h after TRAIL addition), apoptosis was detected by Annexin V binding (A) and as subG1 peak in DNA content measurement (B).

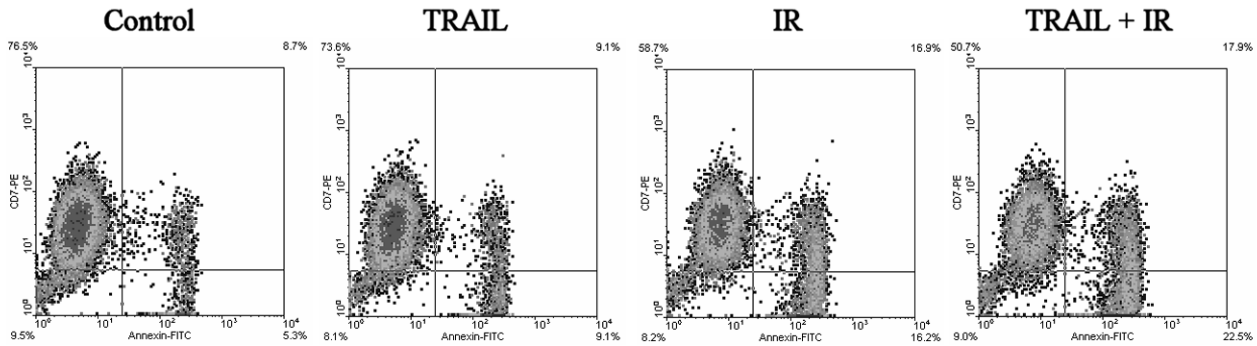

Fig. 5: Dual detection of CD7 and Annexin V binding.
MOLT-4 cells were irradiated by the dose of 1 Gy and TRAIL in final concentration 100ng/ml was added to the culture medium 16 h after the irradiation. 22 h after the irradiation the cells were analyzed for Annexin V binding and CD7.

Death receptors ligands such as TRAIL have the ability to induce death in susceptible cell types, which arouses considerable interest in their therapeutical potential as antitumor drugs. TRAIL is selectively toxic to a variety of human tumor cells, and TRAIL-based agents recently entered clinical trials (5). Primary leukemia cells and established leukemia cell lines studied to date have shown variable susceptibility to TRAIL. Wen et al. (17) and our previous work (13) prove that in acute leukemia cells HL-60, U 937 and Jurkat TRAIL activates intrinsic mitochondrial pathways of apoptosis. Since the cells lack a functional p53, this activation must be p53 independent. It was also shown that p53 regulates transcription of proapoptotic receptor DR5. Although the Apo-2L-sensitive acute leukemia cell types expressed DR5 and in HL-60 also DR4, the level of expression of these death-signaling receptors did not correlate with the sensitivity to TRAIL (17). The cells can be TRAIL-resistant not only due to low expression of DR-4/5, but also due to increased expression of caspase activation inhibitor FLIP (8). Sensitivity to TRAIL is also dependent on the amount of antiapoptic proteins from the Bcl-2 family: increased expression of Bcl-2 a Bcl-x_L inhibits TRAIL-induced apoptosis (6). Defects in gene expression in many tumors (e.g. CLL has a high frequency of Bcl-2 overexpression) are responsible for increased resistance of these cells to apoptosis induction. In HL-60 cells (high expression of

DR5 and DR4), TRAIL application also increases radiosensitivity because it leads to a decrease in antiapototic Mcl-1 (13).

In this work we demonstrate high resistance of MOLT-4 cells to TRAIL, and according to the levels of TRAIL receptor DR5 in the membrane of the cells we presume that the resistance is due to low expression of DR5.

Anitleukemic drugs that cause DNA damage and trigger intrinsic pathways of apoptosis, such as etoposide, Ara-C and doxorubicine, increase DR5 levels in tumor cells, including acute leukemia cell lines (17). One of the less common approaches in treatment of hematological malignancies is low-dose total body irradiation. The standard schedule for treatment of non-Hodgkin lymphomas consisted of giving 0.1–0.25 Gy per fraction, 1–5 times a week to a total dose of 1.5–2 Gy (14). IR damages DNA by a broad spectrum of lesions, of which the most lethal are DSB. IR is a well known activator of intrinsic apoptotic pathways. In this work we prove that the level of DR5 increases in MOLT-4 cells after irradiation using doses of 1 and 3 Gy. The increase is apparent 6 h after the exposure to IR and lasts at least 24 h. These data are supported by findings of Gong and Almasan (4), who detected an increase in DR5 mRNA in MOLT-4 cells 8 h after irradiation by a lethal dose of 10 Gy. Application of TRAIL in the time of increased DR5 positivity increases apoptosis induction despite the original resistance of MOLT-4 cells to TRAIL. Apoptosis induction is also accompanied by loss of CD7 surface marker.

Our study demonstrates that IR in the therapeutically achievable dose of 1 Gy sensitizes TRAIL-resistant cells of acute T-lymphoblastic leukemia MOLT-4 to the TRAIL-induced apoptosis by increasing in death receptors for TRAIL DR5. When TRAIL is applied in the time of increased DR5 positivity, more efficient cell killing is achieved.

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