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The importance of abrogation of G_2-phase arrest in combined effect of TRAIL and ionizing radiation

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Background: In this work we studied the relationship between the enhanced expression of DR5 receptor and the effect of combination of TRAIL and ionizing radiation on cell cycle arrest and apoptosis induction in human leukemia cell line HL-60. Material and methods: DR5, APO2.7 and cell cycle were analyzed by flow cytometry. Proteins Bid and Mcl-1 were analyzed by Western-blotting. For clonogenic survival, colony assay on methylcellulose was used. Results: Ionizing radiation caused significantly enhanced positivity of DR5 receptors 24 h after irradiation with high doses (6 and 8 Gy). An increase of DR5 receptor positivity after a dose of 2 Gy was not statistically significant and application of TRAIL 48 h after irradiation did not increase the apoptosis induction. However, a decrease of radiation-induced G_2 phase arrest and an increase of apoptosis were observed when TRAIL was applied 16 h before irradiation with the dose of 2 Gy. Incubation with 6 μg/l TRAIL for 16 h reduced D_0 value from 2.9 Gy to 1.5 Gy. The induction of apoptosis by TRAIL was accompanied by Bid cleavage and a decrease of antiapoptotic Mcl-1 16 h after incubation with TRAIL. Conclusion: TRAIL in concentration of 6 μg/l applied 16 h before irradiation by the dose of 1.5 Gy caused the death of 63% of clonogenic tumor cells, similarly as the dose of 2.9 Gy alone, which is in good correlation with the enhanced apoptosis induction.

Keywords: TRAIL, ionizing radiation, DR5 receptor, apoptosis, HL-60 cells

TRAIL is a potent activator of cell death acting through so-called „death receptors" DR4 and DR5. TRAIL preferentially kills tumor cells while it is non-toxic towards most of normal tissues. Plasilova *et al.* (2002) have shown that TRAIL does not have any negative effect on the number of CFU-GM colonies and clusters derived from bone marrow cells of AML patients in complete remission and lymphoma patients without bone marrow involvement, as well as cells derived from normal cord blood. On the other hand, it suppressed the growth of early primary leukemia and myelodysplasia progenitors.

The receptors for TRAIL, DR4 and DR5, contain a cytoplasmic "death-domain" capable of engaging the cell suicide apparatus through an adaptor molecule intermediate such as a Fas-associated death domain protein (Srivastava, 2001). The main pathway which activates the apoptotic process started by death receptors leads through death inducing signaling complex (DISC) assembly and activation of caspase 8 and further activation of caspase 3 (Gong & Almasan, 2000). However, the apoptotic process can be also activated by TRAIL through the mitochondrial pathway. In such case, Bid (Bcl-2 inhibitory BH3-domain-containing protein) is cleaved by caspase 8, the apoptosome is assembled, caspase 9 is activated, and it further activates effector caspase 3 (Belka *et al.*, 2001). Wen *et al.* (2000) has shown that for cells of hematopoietic lineages Jurkat, HL-60 and U-937 apoptosis induced by TRAIL is related mainly to the mitochondrial pathway, i.e.

Abbreviations: AML, acute myeloid leukemia; ATM, ataxia telangiectasia-mutated; CFU-GM, colony-forming unit granulocyte macrophage; DR, death receptor; FCS, fetal calf serum; HDR, high dose-rate; LDR, low dose-rate; mAB, monoclonal antibody; PBS, phosphate-buffered saline; PE, phycoerythrin; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand.

Bid activation and accumulation of cytochrome *c* in the cytosol. An increased expression of antiapoptotic proteins Bcl-2 and Bcl-X_L significantly reduces apoptosis induction by TRAIL in HL-60 cells. Caspase 8 and 9 inhibitors also inhibit apoptosis induced by TRAIL. Another member of the Bcl-2 protein family is Mcl-1. High level of Mcl-1 in hematopoietic cells is responsible for survival of hematopoietic progenitor cells (Kitada & Reed, 2004). Mcl-1 is one of the very labile proteins of the Bcl-2 family; it contains sequences enabling quick degradation. Expression of Mcl-1 significantly increases after therapy using growth factors and cytokines. Parallels exist between Mcl-1 and Bcl-2, suggesting that Mcl-1 also reacts with Bax and therefore extends the survival of hematopoietic cells in cytotoxic conditions (Zhou *et al.*, 1997).

Several observations indicate that radiation and receptor-induced apoptosis each use distinct although partially overlapping pathways. Efforts to combine ionizing radiation and TRAIL in cancer therapy are therefore reasonable. Ionizing radiation in doses up to 10 Gy causes in HL-60 cells (which lack functional p53) a long G_2 phase arrest and apoptosis is observed as late as 48 h after irradiation (Marekova *et al.*, 2003). Ionizing radiation induces apoptosis by causing double strand breaks of DNA, activation of ATM kinase and by the mitochondrial pathway by activation of proapoptotic members of Bcl-2 family (Bakkenist & Kastan, 2003). Abrogation of G_2 phase arrest, e.g. by caffeine, has a strong radiosensitizing effect in HL-60 cells (Vavrova *et al.*, 2003).

In our work we looked for the most effective sequence of the combination of therapeutic doses of ionizing radiation and TRAIL. The application of TRAIL after irradiation has been supported by some of the previous studies (Belka *et al.*, 2001), proving the increase of receptors containing death domain after irradiation of Jurkat cells. Also application of etoposide, ara-C and doxorubicin increases DR5 receptors in HL-60, Jurkat and U-937 cells. Wen *et al.* (2000) have found that treatment of these cells with the above-mentioned cytostatics for 6 h followed by TRAIL application causes stronger apoptosis induction in comparison to simultaneous treatment. However, Kim *et al.* (2001) have shown that doxorubicin increases the amount of DR5 receptor and enhances TRAIL-induced apoptosis also in normal lung and skin fibroblasts.

In our work we studied the expression of DR5 receptor after irradiation of HL-60 cells and the expression of members of Bcl-2 protein family (Mcl-1 and Bid), which can significantly affect apoptosis induction. We looked for the lowest dose of TRAIL, which in combination with the therapeutic dose of 2 Gy of ionizing radiation, would have a significant effect on apoptosis induction and the loss of clonogenic survival in human promyelocytic leukemia HL-60 cells.

MATERIALS AND METHODS

Cell cultures and culture conditions. Human leukemia HL-60 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). 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 a humidified incubator at 37°C and controlled 5% CO_2 atmosphere. The cultures were divided every 2nd day by dilution to a concentration of 2×10^5 cells/ml. The cell counts were performed with a hemocytometer, the cell membrane integrity was determined by using the Trypan blue exclusion technique. Cell lines at no more than 20 passages were used for this study.

Gamma irradiation. Aliquots of 10 ml of cell suspension (2×10^5/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 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 a buffer containing 20 mM Hepes, pH 7.7, 300 mM NaCl, 0.006% Tween 20, 1% sucrose, 0.5 mM DTT (dithiothreitol).

Cell cycle analysis. The cells were collected and washed with cold PBS (phosphate-buffered saline), and fixed in 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 propidium iodide (PI) in the Vindelov's solution for 30 min at 37°C. The fluorescence (DNA content) was measured with a Coulter Electronic (Hialeah, FL, USA) apparatus. A minimum of 10000 cells analyzed in each sample served to determine the percentages of cells in each phase of the cell cycle, using the Multicycle AV software. Three independent experiments were performed.

***In vitro* clonogenic survival assay**. The radiation survival curves were generated using an *in vitro* clonogenic assay. The HL-60 cells (10^2–10^5/ml) were mixed in the Iscove's modified Dulbecco's medium supplemented with 0.9% methylcellulose, 30% fetal calf serum and 10% conditioned medium from the 5637 human bladder carcinoma cell line. One mil-

lilitre of the plating mixture was dispersed into 35 mm tissue culture Petri dishes. The colonies (containing 40 or more cells) were counted after 14 days of incubation in 5% CO_2 and 5% O_2 at 37ºC and the curves were generated. All semi-solid cultures were performed in duplicates. Two independent experiments (4 measurements) were performed.

Flow cytometry. The cells were washed twice with PBS containing 5% FCS and 0.02% NaN_3. Then, 1×10^5 cells suspended in 0.5 ml of PBS with 5% FCS and 0.02% NaN_3 were incubated with mAbs:

1. APO2.7 antibody: For the apoptosis detection mouse phycoerythrin (PE)-conjugated mAb APO2.7 clone 2.7 A6A3 (obtained from Immunotech, Prague, CR) specific for a 38 kDa mitochondrial protein (7A6 antigen) was used. The cells were incubated with mAbs for 30 min at 4ºC. The method with nonpermeabilised cells was used.

2. Antibody to DR5: For detection of TRAIL receptor DR5 mAb 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.

All flow cytometric analyses were 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 10000 cells was collected for each sample in a list mode file format. The list mode data were analyzed using Epics XL System II software (Coulter Electronic, Hialeah, FL, USA). Three independent experiments were performed.

Electrophoresis and Western blotting. At various times after irradiation, the HL-60 cells were washed with PBS and lysed. Whole cell extracts were prepared by lysis in 500 μl of lysis buffer (137 mM NaCl, 10% glycerol, 1% n-octyl-β-D-glucopyranoside, 50 mM NaF, 20 mM Tris, pH 8, 1 mM Na_3VO_4, Complete ™Mini). The lysates containing equal amount of protein (30 μg) were loaded onto a 12% SDS/polyacrylamide gel. After electrophoresis, proteins were transferred to a PVDF membrane and hybridized with an antibody (rabbit anti-Bid polyclonal antibody from PharMingen, 1:500, and anti-Mcl-1 antibody developed in rabbit by Sigma-Aldrich, 1:1500). After washing, the blots were incubated with secondary peroxidase-conjugated antibody diluted 1:1000 (Dako, High Wycombe, UK) and the signal was developed with a chemiluminescence (ECL) detection kit (Boehringer Mannheim).

Statistical analysis. The results were statistically evaluated with Student's *t*-test. The values represent the mean ±S.D. (standard deviation of the mean). The statistical significance of the difference of means in comparable sets is indicated.

The statistical evaluation of the clonogenic survival curves was performed as follows: At first clonogenic survival curves were approximated by linear combination of orthogonal polynomials. Together the highest polynomial degree was determined with the help of statistical test by testing of appropriate regression coefficient against zero; the test was rejected on significance level α = 5% (Ralston, 1973). Statistical evaluation of mutual (un)identity of clonogenic survival curves was provided with help of two-dependence (two-sided) *t*-test. It was found out from *P*-value dependence on the dose or concentration in which dose or concentration range the clonogenic survival curves do not mutually statistically differ and in which domains they are mutually statistically different.

RESULTS

DR5 receptors after irradiation

As the main goal of this study was to find the most effective sequence for combination of TRAIL and ionizing radiation, we evaluated changes in expression of TRAIL DR5 receptors after exposure of HL-60 cells to different doses of ionizing radiation. As shown in Fig. 1, ionizing radiation induced a significant increase of DR5 receptors after irradiation with the doses of 6 and 8 Gy, as detected by flow cytometry 24 h after irradiation. On the other hand, the therapeutically used dose of 2 Gy (a dose usually used as one fraction during fractionated radiotherapy) did not significantly increase DR5 receptors.

Combination of ionizing radiation followed by TRAIL

As the most pronounced (albeit nonsignificant) increase of TRAIL DR5 receptors was detected 48 h after irradiation with the dose of 2 Gy, we decided to add TRAIL to the culture medium at this time. The cells were irradiated with the dose of 2 Gy and 48 h later TRAIL was added to culture medium in a final concentration of 20 μg/l. However, no significantly greater effect of the combination treatement was obtained (not shown).

Combination of TRAIL treatment followed by ionizing radiation

In our further experiments we observed an effect of a 16-h-long incubation with TRAIL and a consecutive irradiation by the dose of 2 Gy on HL-60 cells. HL-60 cells were incubated for 16 h in the presence of TRAIL (5–100 μg/l). When TRAIL had

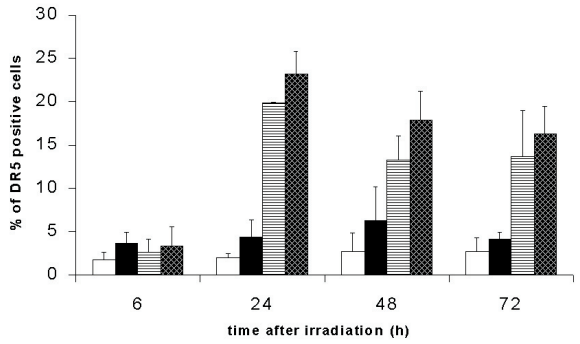

Figure 1. The effect of irradiation of HL-60 cells on positivity of TRAIL receptor DR5.
The cells were irradiated by the dose of 2, 6 and 8 Gy of ionizing radiation and the positivity of DR5 receptors was determined by flow cytometry 6, 24, 48 and 72 h after the irradiation. Each point represents the average of three independent experiments ±S.E.M. ❑, Control; ■, 2 Gy; ▒, 6 Gy; ❖, 8 Gy.

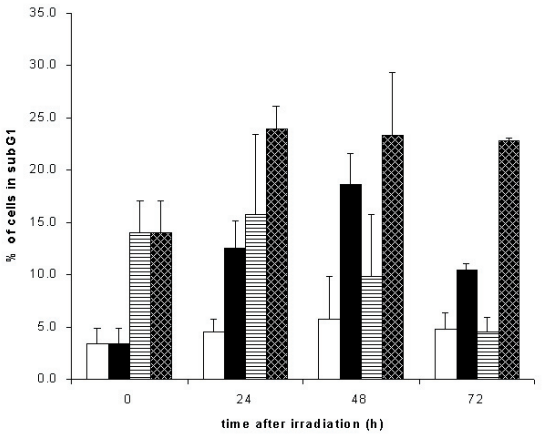

Figure 2. The comparison of effect of the 16-h-long incubation with 6 μg/l TRAIL, the irradiation by the dose of 2 Gy, and their combination on apoptosis induction (detection of subG$_1$ cells during DNA content analysis) in HL-60 cells.
TRAIL was applied 16 h before irradiation and washed away immediately after irradiation. Cells in early phase of apoptosis are detected as subG$_1$ peak (i.e. cells with lower amount of DNA than cells in G$_1$ phase of cell cycle) due to fragmentation and loss of DNA during apoptosis. Each point represents the average of three independent experiments ±S.E.M. ❑, Control; ■, 2 Gy; ▒, TRAIL 6 ng/ml; ❖, combination.

been washed away, one group was irradiated by the dose of 2 Gy. Immediately after the irradiation the clonogenity test was started and the number of colonies was determined on 14th day of cultivation. As far as the clonogenic survival of HL-60 cells is concerned, the application of TRAIL 16 h before irradiation with the dose of 2 Gy had an additive effect. The concentration of TRAIL which caused a decrease of the colony number to 50% (EC_{50}) was 19.1 μg/l for nonirradiated cells. In the case of cells irradiated with the dose of 2 Gy after 16 h of incubation with TRAIL, the EC_{50} value decreased to 6.3 μg/l. From the P(c) behavior it was observed that in the concentration range from 0 to 35.4 μg/l, $P(c) \leq 0.01$, and therefore both tabular dependencies may statistically be considered as highly different; in the concentration range 35.4 to 45.2 μg/l, where $0.01 < P(c) \leq 0.05$, as different, and within the concentration range from 45.2 to 51.9 μg/l, where $0.05 < P(c) \leq 0.1$, as marginally different.

In the following experiments we studied the effect of a 16-h-long incubation with 6 μg/l TRAIL (concentration close to EC_{50} when combined with 2 Gy irradiation, as mentioned above) combined with the irradiation with the dose of 2 Gy on the mode of cell death and cell cycle progress. Apoptosis was detected by two different methods — subG$_1$ peak (Fig. 2) and APO2.7 positivity (Fig. 3). The method analyzing the subG$_1$ peak detects early phase of apoptosis, while the method analyzing the mitochondrial antigen APO2.7 in nonpermeabilised cells detects a later phase of apoptosis (Marekova *et al.*, 2003). We proved that the combination (TRAIL followed after 16 h by 2 Gy irradiation) is more effective in induction of apoptosis, which was more intensive and lasted longer after irradiation.

When looking for the mechanism involved in the enhanced apoptosis induction we found that the application of TRAIL inhibited the cell cycle arrest in G$_2$ phase, which is observed 24 h after irradiation with the dose of 2 Gy. A representative result of one out of three experiments is shown in Fig. 4. There was a statistically significant decrease of the percentage of cells in G$_2$ phase 24 h after TRAIL and irra-

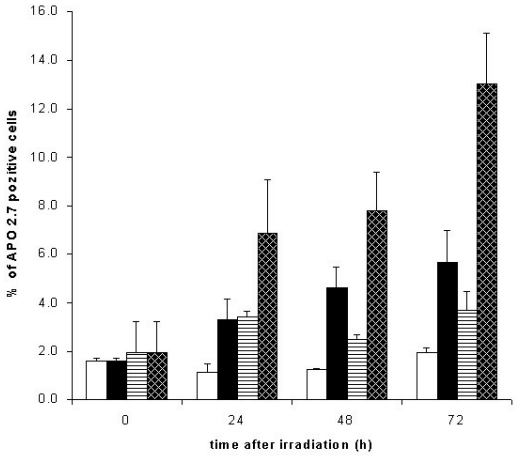

Figure 3. The comparison of effect of the 16-h-long incubation with 6 μg/l TRAIL, the irradiation by the dose of 2 Gy, and their combination on apoptosis induction (detection of mitochondrial membrane antigen APO2.7, without permeabilization) in HL-60 cells.
TRAIL was applied 16 h before irradiation and washed away immediately after irradiation. Mitochondrial membrane antigen APO2.7 is a specific marker of apoptosis. When used without digitonin permeabilization of cell membrane it detects cells in later phase of apoptosis. Each point represents the average of three independent experiments ±S.E.M. For designations see Fig. 2.

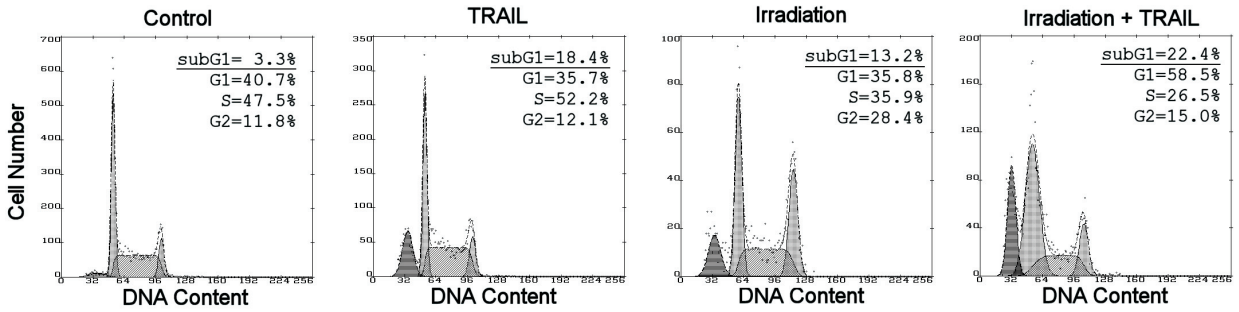

Figure 4. The effect of 16-h-long incubation with TRAIL (6 μg/l) and consecutive irradiation by the dose of 2 Gy on abrogation of cell cycle arrest in G_2 phase.
TRAIL was applied 16 h before irradiation and washed away immediately after irradiation. The cells were analyzed 24 h after irradiation. The percentage of $subG_1$ cells is calculated from the total cell count, distribution of the cells in cell cycle (the percentage of cells in G_1, S, G_2) is calculated only among the cells in the cycle (excluding $subG_1$). Representative results of one out of three independent experiments.

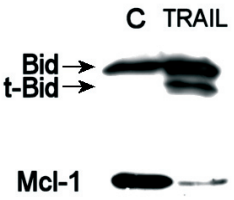

Figure 5. TRAIL induced changes in proteins of Bcl-2 family.
HL-60 cells were incubated with TRAIL (100 μg/l) for 16 h. Bid and Mcl-1 proteins were detected by Western blotting. TRAIL induced increase of truncated form of Bid (15 kDa) and decrease of Mcl-1 levels.

diation (13.9 ± 1.2%) in comparison to the irradiated only (2 Gy) group (27.2 ± 6.3%).

We next proved that a 16-h-long incubation with TRAIL induces changes in Bid and Mcl-1, proteins of the Bcl-2 family, in HL-60 cells. Using Western blot analysis we detected induction of the pro-apoptotic form of Bid (t-Bid) and inhibition of the anti-apoptotic protein Mcl-1 (Fig. 5).

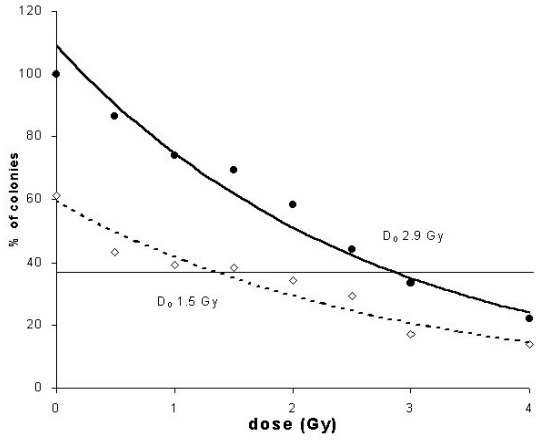

Figure 6. The effect of 16-h-long incubation with 6 μg/l TRAIL and consecutive irradiation by the dose of 2 Gy on clonogenic survival of HL-60 cells.
For the clonogenic survival data, each point is a mean of 4 measurements from two experiments ± S.D. The clonogenicity test has been started immediately after the end of the irradiation. Equations of generated curves: Control: $y = 109.22e^{-0.3781x}$, TRAIL: $y = 59.711e^{-0.3519x}$. ●, Control; ◇, TRAIL.

Effect of TRAIL on radiosensitivity of HL-60 cells

Incubation with 6 μg/l TRAIL for 16 h reduced the D_0 value (the dose of ionizing radiation after which 37% of clonogenic HL-60 cells survive) from 2.9 Gy to 1.5 Gy. From the *P*(c) behavior it was observed that in the dose range from 0 to 2.77 Gy, $P(c) \leq 0.01$, and therefore both tabular dependencies may be considered as statistically highly different; in the dose range 2.77 to 2.98 Gy, where $0.01 < P(c) \leq 0.05$, as different, and within the dose range 2.98 to 3.09 Gy, where $0.05 < P(c) \leq 0.1$, as marginally different. The preincubation of HL-60 cells with TRAIL did not change the radiosensitivity itself, but shifted the dose-response curves due to the additional killing of the cells by TRAIL (Fig. 6).

DISCUSSION

Cancer cells often develop a resistance to chemotherapy or irradiation through mutations in the p53 tumor-suppressor gene, which prevent apoptosis induction in response to cellular damage. The HL-60 cells (devoid of p53) are more radioresistant in comparison to the human T-lymphocyte leukemia cells MOLT-4 (wild type p53), mainly due to the long reparation of radiation-induced damage during the G_2 phase arrest of the cell cycle. Irradiation of HL-60 cells by a low dose-rate gamma radiation (0.23 Gy/h, LDR) caused prolonged irradiation in G_2 phase and increased radioresistance in comparison to a high dose-rate irradiation (0.6 Gy/min, HDR). The cells repaired their damage already during the LDR irradiation (Vavrova *et al.*, 2004). When the HL-60 cells were irradiated by a low dose-rate gamma

radiation in the presence of the ATM kinase inhibitor caffeine, the D_0 value decreased from 3.7 Gy to 2.2 Gy (LDR in the presence of caffeine) (Vavrova *et al.*, 2003).

The effect of therapeutic doses of ionizing radiation on tumor cells can be enhanced by combininig ionizing radiation with other substances which selectively enhance apoptosis induction in these cells. One of such selective inducers of apoptosis is TRAIL. From our and others (Wen *et al.*, 2000) results it is apparent that TRAIL induces apoptosis regardless of p53 status. We proved that in the range of low doses (up to 4 Gy) the application of 6 μg/l TRAIL 16 h before irradiation significantly reduces the D_0 value from 2.9 Gy to 1.5 Gy. This observation is in good correlation with the results showing that application of low concentration of TRAIL prevents the accumulation of the cells irradiated by the dose of 2 Gy in G_2 phase of the cell cycle, and therefore disables the reparation of radiation-induced damage, which in turn causes enhanced apoptosis induction. The relatively long interval of detectable apoptosis induced by a combination of TRAIL and irradiation could be caused by the attempt of the irradiated cells to enter mitosis with an insufficiently repaired DNA, which is responsible for delayed post-mitotic apoptosis in later intervals. Similar results were obtained during studies of the combined effect of caffeine applied 30 min before irradiation (Vavrova *et al.*, 2003).

The importance of the mitochondrial pathway of apoptosis induction after TRAIL seems to be beyond doubts in the case of leukemic cells. Belka *et al.* (2001) have shown the activation of Bid 4–6 h after application of TRAIL in Jurkat cells. In our work we detected an active form of Bid and a significant decrease of the antiapoptotic Mcl-1 16 h after treatment of HL-60 cells with TRAIL.

The interactions of TRAIL and radiation were studied in the HL-60 cell line, because these cells are sensitive to apoptosis induced by TRAIL alone and they are less sensitive to ionizing radiation (D_0 = 2.2 Gy) in comparison to hematopoietic stem cells (D_0 = 1 Gy) (Vavrova *et al.*, 2002). We proved a dose dependent up-regulation of DR5 receptor after the studied doses of radiation (6 and 8 Gy). After the dose of 2 Gy the expression of DR5 receptor increased (but not significantly) only 48 h after irradiation. When TRAIL was applied 48 h after irradiation of the cells by the dose of 2 Gy we did not detect a higher amount of apoptosis in comparison to nonirradiated cells. Belka *et al.* (2001) have described an increase of DR5 receptor expression in Jurkat cells 12 and 24 h after the dose of 10 Gy. A 6-h-long incubation of HL-60 cells with increasing concentrations of ara-C (1–100 μmol/l) also significantly increased the DR5 receptor amount. However, even the dose of 8 Gy did not cause an increase of DR5 receptors during 6 h after irradiation in our experiments with HL-60 cells. The expression of DR5 receptors increased also after 6-h-long incubation of HL-60 cells with 50 μmol/l doxorubicin (Wen *et al.*, 2000). It seems to be apparent that both ara-C and doxorubicin are better inducers of the DR5 receptor expression in comparison to ionizing radiation. On the other hand, doxorubicin was also found to sensitize normal nontumor lung and skin fibroblasts to TRAIL (Kim *et al.*, 2001). When we applied TRAIL 48 h after irradiation by the dose of 2 Gy (i.e. during the period of the highest expression of DR5 receptors after this dose of radiation) we did not detect an increased apoptosis after TRAIL in comparison to the nonirradiated group.

We proved in this work that the incubation of HL-60 cells with TRAIL 16 h before irradiation prevented the accumulation of cells in the G_2 phase after irradiation and induced a higher degree of apoptosis of irradiated cells. This resulted in a synergic effect, where the EC_{50} value decreased from 19.1 μg/l (TRAIL) to 6.3 μg/l (TRAIL + irradiation by the dose of 2 Gy). The results of Kim *et al.* (2001) proved that TRAIL-resistant Jurkat clones were not cross-resistant to ionizing radiation. The combined treatment of radiation and TRAIL synergistically increased the cell death through the activation of the caspase pathway in clones resistant to TRAIL-induced apoptosis. When TRAIL and ionizing radiation were applied together no increase of DR5 or DR4 receptors was detected. This means that the synergic effect was caused by TRAIL receptor-independent activation of the caspase 8 cascade pathway. The interaction of irradiation and TRAIL involves multiple genes such as DR5, caspase 8, caspase 3, and Bcl-2 family. Marini *et al.* (2003) proved that the expression of receptor DR5, the adaptor molecule FADD and initiator caspase 8 or 10 is required for the combined effect of TRAIL and irradiation. FADD is not necessary for the ionizing radiation effect, but it is absolutely essential for the TRAIL-induced apoptosis. FADD negative cells show no additive effect of radiation and TRAIL.

Procaspase 8 seems to be the key molecule in the apoptosis induced through death receptors. Its function can be partially substituted by caspase 10. The down-regulation of Bcl-2 and Mcl-1 also plays an important role in apoptosis induction. We proved that after a 16-h-long incubation of HL-60 cells with TRAIL the active form of Bid appears and the amount of Mcl-1 significantly decreases, which can also contribute to a stronger effect of ionizing radiation.

We proved that the anti-tumor effect of TRAIL applied 16 h before irradiation of HL-60 cells (p53 negative) was linked with the higher intensity of apoptosis induction and with the abrogation of the G_2 phase arrest induced by ionizing radiation.

We also showed that the therapeutically used dose of 2 Gy did not significantly increased DR5 receptor positivity of HL-60 cells. The combined effect of irradiation (2 Gy) and TRAIL (6 μg/l) is synergic mostly because the application of TRAIL before irradiation prevents the cells from accumulating in G_2 phase, and consequently they cannot repair the radiation-induced damage sufficiently. This causes enhanced apoptosis induction, which is also demonstrated in the decrease of the D_0 value from 2.9 Gy to 1.5 Gy.

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REFERENCES

Bakkenist CJ, Kastan MB (2003) DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* **421:** 486–488**.**

Belka C, Schmid B, Marini P, Durand E, Rudner J, Faltin H, Bamberg M, Schulze-Osthoff K, Budach W (2001) Sensitization of resistant lymphoma cells to irradiation--induced apoptosis by the death ligand TRAIL. *Oncogene* **20:** 2190–2196.

Gong B, Almasan A (2000) Apo2 ligand/TNF-related apoptosis-inducing ligand and death receptor 5 mediate the apoptotic signaling induced by ionizing radiation in leukemic cells. *Cancer Res* **60:** 5754–5760.

Kim MR, Lee JY, Park MT, Chun YJ, Jang YJ, Kang CM (2001) Ionizing radiation can overcome resistence to TRAIL in TRAIL-resistant cancer cells. *FEBS Lett* **505:** 179–184.

Kitada S, Reed JC (2004) Mcl-1 promoter insertions dial-up aggressiveness of chronic leukemia. *J Natl Cancer Inst* **96:** 642–643.

Marekova M, Vavrova J, Vokurkova D (2003) Monitoring of premitotic and postmitotic apoptosis in gamma irradiated HL-60 cells by the mitochondrial membrane protein-specific monoclonal antibody APO2.7. *Gen Physiol Biophys* **22:** 191–200.

Marini P, Jendrossek V, Durand E, Gruber C, Budach C, Belka C (2003) Molecular requirements for the combined effects of TRAIL and ionizing radiation. *Radiother Oncol* **68:** 189–198.

Plasilova M, Zivny J, Jelínek J, Neuwirtova R, Čermák J, Necas E, Andera L, Stopka T (2002) TRAIL (Apo2L) suppresses growth of primary human leukemia and myelodysplasia progenitors. *Leukemia* **16:** 67–73.

Ralston A (1973) *A First Course in Numerical Analysis***.** McGraw Hill Book Comp, New York, St. Louis, San Francisco, Toronto, London, Sydney.

Srivastava RK (2001) TRAIL/APO-2L: Mechanism and clinical applications in cancer. *Neoplasia* **3:** 535–546.

Vavrova J, Vokurkova D, Marekova M, Blaha M, Jebavy L, Filip S (2002) Antiapoptotic cytokine IL-3 + SCF + FLT3L influence on proliferation of gamma-irradiated $AC133^+/CD34^+$ progenitor cells. *Folia Biol* **48:** 51–57.

Vavrova J, Marekova-Rezacova M, Vokurkova D, Szkanderova S, Psutka J (2003) Caffeine induces a second wave of apoptosis after low dose-rate gamma radiation of HL-60 cells. *Radiat Environ Biophys* **42:** 193–199.

Vavrova J, Rezacova M, Vokurkova D, Psutka J (2004) Cell cycle alteration, apoptosis and response of leukemic cell lines to gamma radiation with high- and low-dose rate. *Physiol Res* **53:** 335–342.

Wen J, Ramedevi N, Nquyen D, Perkins C, Worthington E, Bhalla K (2000) Antileukemic drugs increase death receptor 5 levels and enhance Apo-2L-induced apoptosis of human acute leukemia cells. *Blood* **96:** 3900–3906.

Zhou P, Qian L, Kozopas KM, Craig RW (1997) Mcl-1, a Bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. *Blood* **89:** 630–643.