Exposure to fractionated dose of 60 Gy affects molecular response of HL-60 cells to irradiation

Jiřina Vávrová¹, Aleš Tichý¹, Martina Řezáčová², Lenka Zárybnická¹, Zuzana Šinkorová¹ and Jan Österreicher¹

¹ Department of Radiobiology, Faculty of Health Sciences, Hradec Králové, University of Defence Brno, Czech Republic
² Institute of Medical Biochemistry, Faculty of Medicine in Hradec Králové, Charles University in Prague, Czech Republic

Abstract. In this work we evaluated changes in molecular response of human promyelocyte leukemia cells HL-60 and HL-60-IR cells (HL-60 irradiated by 10 cycles of radiation with total dose of 60 Gy, given over a period of 3 months) to irradiation by the dose of 2 and 8 Gy. Analysis of CD11b and apoptosis by flow-cytometry revealed that on 3rd day after irradiation by 8 Gy the HL-60-IR are more resistant to radiation-induced apoptosis and more differentiated (increase in CD11b in non-apoptotic cells) than regular HL-60.

We found that both types of cells have high basal level of phosphorylated extracellular signal-regulated kinases Erk1/2. Irradiation induces decrease in Erk1/2 phosphorylation after 4 and 8 h in both cell types. However, in HL-60-IR cells Erk1/2 phosphorylation is restored faster than in HL-60. Also it was found that in contrary to HL-60 cells, the HL-60-IR cells react to 2 Gy irradiation by p53 independent increase in p21(WAF1/Cip1), and not by activation of checkpoint kinase Chk-2.

Therefore we concluded that relatively high dose of radiation (6 Gy) does not lead after 10 repetitive irradiations to eradication of HL-60 cells, but instead increases their radioresistance, increases the ability to differentiate, alters MAPK response, increases amount of p21(WAF1/Cip1), and decreases induction of apoptosis by ionizing radiation. p21(WAF1/Cip1) might prevent apoptosis induction and trigger permanent cell-cycle arrest, which can also contribute to regression of this leukaemia after therapy.

Key words: Ionizing radiation — Radioresistance — p21(WAF1/Cip1) — Erk1/2 — Apoptosis

Abbreviation: HL-60, human promyelocyte leukemia cell line; HL-60-IR, HL-60 irradiated by 10 cycles of radiation with total dose of 60 Gy, given over a period of 3 months; CEM/IR, CEM leukaemia cell line irradiated by 10 cycles of radiation with total dose of 60 Gy; MAPK, mitogen-activated protein kinase; Erk1/2, extracellular signal-regulated kinases; p38, mitogen-activated protein kinase; Jnk, c-Jun NH2-terminal kinase; DNA-PK, DNA-dependent protein kinase; Chk-2, checkpoint kinase; p53, tumor suppressor protein p53; p21, cyklin-dependent kinase inhibitor p21(WAF1/Cip1); TGFα, transforming growth factor α; TNFα, tumor necrosis factor α; Mcl-1, myeloid cell leukemia-1 protein; NF-κB, nuclear-factor-κB; ATRA, all-trans retinoic acid.
largely associated with apoptosis, whereas signalling via the extracellular signal-regulated kinases pathways (Erk) is primarily linked with the cell proliferation and survival, and seems to prevent apoptosis (Golding et al. 2007). Also ionizing radiation and other toxic stressors lead to simultaneous compensatory activation of multiple MAPK pathways. Low doses of radiation activate Erk1/2 and p38 in normal lung fibroblasts (Kim et al. 2007). The expression and release of autocrine growth factor ligands such as TGFe or TNFα also increase the response of MAPK pathway. Enhancement of basal signalling by proto-oncogene such as K/H/N-Ras may provide a radioprotective and growth-promotion signal (Dent et al. 2003). Dent et al. (1999) show that induction of MAPK pathway has cytoprotective effect against ionizing radiation. On the other hand, short activation of MAPK cascade can increase cell proliferation, while long activation of MAPK can cause inhibition of the DNA synthesis via super-induction of p21 (Park et al. 1999). Some papers show that in cells without functional p53 radiation triggers epidermal growth receptors pathways, leading to increase in p21 and cell cycle arrest in both G1/S and G2/M phase. Taken together, radiation-activated MAPK signalling and p21 play an important role in regulation of cell cycle progress. The ability of a cell to express p21 is decreased by inhibition of MAPK pathway. Therefore, inhibition of MAPK pathway increases cell death by apoptosis after irradiation (Carter et al. 1998).

Radioresistance of tumour cells can also be caused by deregulation of DNA damage repair. DNA-PK is a serine/threonine kinase involved in DNA damage recognition and repair. DNA-PK consists of DNA-PK catalytic subunit (DNA-PKcs) and two subunits Ku70 and Ku80. This kinase is involved in repair of double-strand breaks of DNA by non-homologous end-joining. Kinase activity of DNA-PK is generally stimulated upon binding of the Ku components to damaged DNA. Shen et al. (1998) describe that the adriamycin resistance of HL-60 cells is accompanied by increase of DNA-PK. On the other hand, increased DNA-PK was not found in cell lines resistant to anti-mitotic drugs such as taxol. This suggests that altered DNA-PK is not a general characteristic of resistant cells and may be consistent with the role of DNA-PK in DNA repair. Corresponding results were obtained by Chin et al. (2005), who proved that radioresistance of leukaemia cells CEM/IR is caused by increased amount of repair proteins DNA-PKcs, Ku70/80, Rad51, and Rad54.

Another possibility is resistance to apoptosis induction. Resistance to apoptosis induction is related to increased amount of anti-apoptotic proteins of Bcl-2 family. This is the case mainly in human lymphomas, where anti-tumour therapy causes resistance to apoptosis induction due to increased expression of Bcl-2. Main physiologic role of Bcl-2 and Bcl-Xl proteins is inhibition of mitochondrial apoptosis pathways. Both molecules inhibit release of pro-apoptotic molecules from mitochondria. Increased expression of Bcl-2 in Jurkat cells completely blocks activation of caspases and apoptosis induction by ionizing radiation (Belka et al. 2000).

One more factor is discussed in relation to radioresistance of tumour cells, and that is NF-kB, a stress sensitive heterodimeric transcription factor in the regulation of the stress-responsive genes, which has been shown to initiate the pro-survival signalling pathways. Ahmed et al. (2006) proved that in human breast cancer cells MCF-7 repeated irradiation by the dose of 6 Gy until total dose of 60 Gy causes increase in NF-kB. Increase in NF-kB subunit p65 levels was also associated with a decrease in phosphorylation of Erk1/2 in all radioresistant lines. Thus during the development of adaptation resistance in these cells NF-kB inhibits Erk phosphorylation to enhance cell survival.

In our present work we prove increase in p21 in radio-resistant sub-line of HL-60 cells irradiated by 10 cycles of radiation with total dose of 60 Gy in comparison to normal HL-60 cells after irradiation by the dose of 2 and 8 Gy. Also the role of MAPK pathway in radioresistance after repeated irradiation is studied and discussed.

Materials and Methods

Cell culture and culture conditions

HL-60 cells were obtained from European Collection of Animal Cells Cultures (Porton Down, Salisbury, UK). The cells were cultured in Iscove’s modified Dulbecco’s medium (Sigma) supplemented with 20% fetal calf serum (PAA Laboratories GmbH, Austria), 2 mM glutamine (Sigma), 100 U/ml penicillin (Sigma) and 0.1 mg/ml streptomycin (Sigma) at 37°C, controlled 5% CO2 and humidified atmosphere. The cultures were divided every second day by dilution to a concentration of 2 × 105 cells/ml. Cell counts were detected 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.

The HL-60 cells were irradiated with 6 Gy fraction per cycle using 60Co gamma radiation source at the dose rate 1 Gy/min and rested for 2 weeks between cycles. Ten cycles of radiation with a total dose of 60 Gy were given over a period of 3 months to produce the HL-60-IR sub-line.

Gamma irradiation

The exponentially growing HL-60 and HL-60-IR cells have been suspended at a concentration of 2 × 106 cells/ml in a complete medium. Aliquots of 10 ml have been plated into 25 cm2 flasks (Nunc) and irradiated using a 60Co gamma-ray
Radioresistant HL-60 cells

source with a dose-rate of 1 Gy/min. After irradiation the flasks have been placed in the incubator and the aliquots of the cells have been removed at various times after irradiation for analysis.

Flow cytometric analysis and apoptosis detection

The apoptosis was measured together with cell surface marker of differentiation – CD11b. For this purpose a two-colour (FITC/PE) flow cytometric analysis was used. The apoptotic cells were detected by Apoptest-FITC kit (DakoCytomation). 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 cell surface marker in HL-60 cells we used PE-conjugated anti-human CD11b (Bear1, IgG1-IM2581, Immunotech). Flow cytometric analysis was performed on the CyAn ADP™ flow cytometric analyzer DakoCytomation (Glostrup, Denmark). Acquisition and analysis were performed using the Summit™ 4.3 software.

Electrophoresis and Western blotting

At various times after irradiation, the HL-60 and HL-60-IR 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-glucopyranoside; 50 mM NaF; 20 mM Tris, pH = 8; 1 mM Na3VO4; 1 tablet of proteases inhibitors Complete™ Mini, Roche, Germany). 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 appropriate antibody: anti-Chk-2 (Thr68), anti-Erk1/2, anti-Erk1/2 (Thr202/Tyr204) from Cell Signalling; anti-lamin B from Oncogene; and anti-p21(WAF/Cip1), anti-Mcl-1, anti-β-actin from Sigma. After washing, the blots were incubated with secondary peroxidase-conjugated antibody (Dako, High Wycombe, UK) and the signal was developed with chemiluminescence detection kit (Boehringer Mannheim) by exposure to film (Foma, Hradec Kralove, CZ).

Statistical analysis

The results have been have statistically evaluated with the Student’s t-test. The values represent mean ± SD (standard deviation of the mean) of minimal three independent experiments.

Results

On the 6th day after irradiation of HL-60 and HL-60-IR cells by the dose of 2 Gy their proliferative capacity was completely restored and HL-60-IR proliferated significantly more than regular HL-60 cells. On the 6th day after irradiation by the dose of 8 Gy the number of cells in both groups was very low and their viability was reduced. The viability after 8 Gy was significantly lower in regular HL-60 (35%) compared to HL-60-IR sub-line (60%) (Fig. 1).
To evaluate induction of differentiation and/or apoptosis the expression of CD11b and phosphatidylserine were analysed 72 h after 2 and 8 Gy irradiation and in the sham treated control by flow cytometry. The results are summarized in Table 1, and representative dot plots are shown in Fig. 2. Irradiation by the dose of 2 Gy induced comparable level of apoptosis in both lines (26 and 23%, respectively). Irradiation by this dose did not induce significant differences in cell lines differentiation. After irradiation by the dose of 8 Gy massive apoptosis (53%) was detected in regular HL-60 cells. Apoptosis induction was significantly lower in resistant HL-60-IR sub-line (31%). Simultaneously, higher differentiation was detected in HL-60-IR sub-line (7%) than in regular HL-60 (2%) (Tab. 1, Fig. 2).

Another method confirming induction of apoptosis in HL-60 and HL-60-IR cells was Western blot analysis of fragmented nuclear protein lamin B. In non-irradiated cells lamin B is presented in the nucleus as a 68 kDa protein. Irradiation causes its cleavage into 46 kDa fragments – products of caspase-catalysed proteolysis during apoptosis. The data obtained from Western blot analysis (Fig. 3) indicate that apoptosis was not detectable after irradiation by the dose of 2 Gy in both cell lines. After irradiation by the dose of 8 Gy lamin B cleavage was detected in HL-60 cells as soon as 8 h and persisted till 144 h after the irradiation, while in resistant HL-60-IR sub-line

<table>
<thead>
<tr>
<th>cells</th>
<th>Annexin V+ (apoptotic) (%)</th>
<th>CD11b+/Annexin V– (differentiated live) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham 2 Gy 8 Gy</td>
<td></td>
</tr>
<tr>
<td>HL-60</td>
<td>8.0 ± 3.4 25.8 ± 7.4 52.8 ± 10.8</td>
<td></td>
</tr>
<tr>
<td>HL-60-IR</td>
<td>8.6 ± 4.4 23.0 ± 2.5 31.0 ± 3.2</td>
<td></td>
</tr>
</tbody>
</table>

Mean values from 3 independent experiments ± S.D.
Radioresistant HL-60 cells

lamin B fragments were detected only later, 72 and 144 h after the irradiation.

Figure 3 compares changes in amount of proteins discussed in the link with radioresistance and their phosphorylation status in HL-60 and HL-60-IR cells.

Total amount of Erk1 and 2 was detected using antibody that detects both isoforms (p44/p42) of the enzyme. No significant changes in total Erk1/2 were observed. Activated phosphorylated form of Erk1/2 was detected by phosphospecific antibody that binds to p44 and p42 isoforms of MAP kinase (Erk1/2) when dually phosphorylated at Thr202/Tyr204 or Thr185/Tyr187. However, only one band was detected by immunoblotting. High phosphorylation of Erk1/2 was detected in both non-irradiated cell lines. This corresponds with rather quick proliferation of both cell lines with doubling time 24 h. After both studied doses of radiation the phosphorylation of Erk1/2 disappears during first hours (4, 8 h) after irradiation. In resistant sub-line HL-60-IR the phosphorylation of Erk1/2 is restored 24 h after irradiation by the dose 2 and 8 Gy, while in regular HL-60 cells the phosphorylation of Erk1/2 is only slightly detected after 72 h and is completely restored only after 144 h. After irradiation by the dose of 8 Gy a slight temporary restoration of Erk1/2 phosphorylation is observed in both cell lines after 24 h, followed again by decrease 72 h after the irradiation. 144 h after the irradiation the phosphorylation is again slightly restored in both cell lines.

Check point kinase 2 was also found to be strongly phosphorylated (Thr68) in regular HL-60 cells within 4 and 8 h after irradiation by the dose 2 and 8 Gy in the contrary to HL-60-IR cells, where this phosphorylation was much less intensive (Fig. 3).

Antiapoptotic protein Mcl-1 is expressed in both cell lines. Its amount increases 4 and 8 h after irradiation by both studied doses (2 and 8 Gy). This initial increase is followed by complete loss of Mcl-1 24 h after the irradiation. After the dose of 2 Gy the protein reoccurs 72 h (HL-60) or 144 h (HL-60-IR) after irradiation (Fig. 3).

Inhibitor of CDK2 protein p21 is not expressed in non-irradiated HL-60 or HL-60-IR cells. In regular HL-60 cells p21 is practically not induced by the dose of 2 Gy. After the dose of 8 Gy the protein is weakly expressed 4 h after irradiation, and it is detected in higher amount 24–144 h after irradiation. In contrast, a very high induction of p21 was detected in resistant HL-60-IR line after irradiation by both doses (2 and 8 Gy) and during the whole interval of the experiment (4–144 h after irradiation) (Fig. 3).

Discussion

Acute myeloid leukaemia is in 40% caused by chromosomal translocation and the TP53 gene is usually mutated or missing. HL-60 cells of human promyelocytic leukaemia do not have TP53 gene, they have normal or slightly increased expression of Bcl-2 and minimal expression of Bcl-XL (Terui et al. 1998). In our previous study (Mareková et al. 2003) we proved two different types of apoptotic cell death after exposure of HL-60 cells to ionizing radiation: premitotic and postmitotic apoptosis. Dose 20 Gy induces quick (4–6 h after irradiation) apoptosis, which is proved by DNA fragmentation and APO2.7 positivity after permeabilization. It is apparent from obtained results that the cells die by apoptosis.

Figure 3. The dynamics of changes of selected proteins in HL-60 and HL-60-IR cells after irradiation by the dose of 2 and 8 Gy. Representative Western blots are shown. HL-60 and HL-60-IR cells were irradiated by the doses 2 and 8 Gy and analysed at 4, 8, 24, 72, and 144 h after the irradiation. Actin–β was used as loading control.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Dose (Gy)</th>
<th>Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erk 1/2</td>
<td>2</td>
<td>0 2 8</td>
</tr>
<tr>
<td>Erk 1/2 _202/204</td>
<td>0 2 8</td>
<td></td>
</tr>
<tr>
<td>Chk-2_68</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Mcl-1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>p21</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Lamin B</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

|          | 2         |          |
|          | 8         |          |

|          | 0         |          |
|          | 2         |          |
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|          | 2         |          |
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|          | 0         |          |
|          | 2         |          |
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|          | 0         |          |
|          | 2         |          |
|          | 8         |          |
from the phase of cell cycle, in which they were irradiated, and that the cells irradiated in G1 phase are less sensitive to this type of apoptosis. After doses lower than 10 Gy, these cells are preferentially arrested in G2 phase and apoptosis occurs lately, 48–72 h after irradiation.

The long cell cycle arrest in G2 phase is responsible for relative radioresistance of these cells, because it allows time for repair of radiation damage (Vávrová et al. 2004). We found no difference in G2/M phase cell cycle arrest after irradiation (2 and 8 Gy) in regular HL-60 and radioresistant HL-60-IR (data not shown) and, therefore, the increased radioresistance of HL-60-IR is not linked with duration of G2 phase arrest.

Another possibility which is discussed in relation to resistance of promyelocytic leukaemia cells to cytostatics and ionizing radiation is the increase in antiapoptotic proteins of Bcl-2 family, or inactivation of proapoptotic protein Bax. In HL-60 cells, high expression of anti-apoptotic protein Mcl-1 was found. Our results show that the amount of Mcl-1 increases 4 and 8 h after irradiation by both studied doses. However, we did not observe any difference between expression of Mcl-1 in regular HL-60 and radioresistant HL-60-IR.

Previously we proved (Rezáčová et al. 2008) that ionizing radiation even causes a pseudo-differentiation in HL-60 cells; the expression of CD11b increases 48–72 h after irradiation. In later intervals, the cells entering apoptosis lose this differentiation antigen. In the present study we show that ionizing radiation (8 Gy) causes higher differentiation (CD11b) and less pronounced apoptosis in resistant sub-line HL-60-IR when compared to reaction of regular HL-60 cells.

All-trans retinoic acid induces the maturation of promyelocytic HL-60 cells towards a neutrophilic phenotype (Watson et al. 1997). MAPK signalling is required for ATRA – triggered G0/G1 cell cycle arrest and cell differentiation; MAPK activation was linked with Erk phosphorylation and p21 accumulation (Wang and Yen 2008). In our experiment, in contrast to regular HL-60 line, a very high induction of p21 is detected in resistant HL-60-IR line after irradiation. During differentiation of cells induced by ATRA, the amount of cell cycle inhibitors increases, mainly of proteins p21 and p27 (Navakauskienė et al. 2002). Increase in the resistance of promyelocytic leukaemia cells to cytostatics and ionizing radiation can also be related to DNA repair processes and expression of DNA-PK. Chin et al. (2005) show that radioresistance of leukaemic cells CEM/IR is caused by increased amount of repair proteins DNA-PKcs, Ku70/80, Rad 51, and Rad 54. However, we found that radioresistant HL-60-IR cells have the amount of DNA-PK comparable to regular HL-60 cells (not published).

Kim et al. (2007) proved that very low doses of radiation (0.05 Gy) lead to increase in phosphorylation of Erk1/2 and p38. This increase can be blocked by specific inhibitors. Abrogation of phosphorylation causes decrease in proliferation of cells. In our work we observed significant decrease in Erk1/2 phosphorylation on Thr202/Tyr204 early after irradiation (4 and 8 h). The phosphorylation was restored much more quickly in resistant HL-60-IR cells in comparison to regular HL-60 cells.

Therefore, we conclude that relatively high dose of radiation (6 Gy) does not lead after 10 (even 20, not shown) repetitive irradiations to eradication of HL-60 cells, but instead increases their radioresistance, increases the ability to differentiate, alters MAPK response, increases amount of p21 and decreases induction of apoptosis by ionizing radiation.

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References
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