Introduction

Acute myeloid leukaemia (AML) accounts for over 80% of all adult acute leukaemias (7) and is a characterised by a clonal expansion of immature myeloid cells in all haematopoietic tissues. Many patients progress to AML from preleukaemic myelodysplastic syndrome (MDS) or from chronic myelogenous leukaemia (CML). AMLs show varied morphologic, cytochemical, immunologic and cytogenetic characteristics and varied sensitivity to conventional chemotherapeutic regimens. Sixty percent to 70% of patients with de novo AML initially achieve complete remission. However, the majority of these patients relapse and eventually die of the disease. The first described and best characterized mechanism of resistance is mdrl gene product, Pglycoprotein. This molecule spans the cell membrane and act as an efflux pump for toxins, including chemotherapy drugs such as anthracyclines, vinca alkaloids and topoisomerase II inhibitors. The biological bases of drug resistance and relapse in AML are not understood and prognoses are still largely based on descriptive parameters. Several lines of evidence indicate that apoptosis plays roles in responses of AML patients to chemotherapy. Aldridge and Radford (1) showed that differences between human haematopoietic cell lines, in the rate of induction of apoptosis after irradiation were generally related to the functioning of cell cycle checkpoints. Whereas the rapidly dying and radiosensitive HSB-2 cell line underwent apoptosis at different points in the cell cycle, the more slowly dying cell lines showed a variety of cell cycle arrest profiles and initiated apoptosis after accumulation of cells in the G2 phase. HL-60 cells showed a markedly longer G2 arrest that correlated with their greater radioresistance. The results suggest that the total length of time available for DNA damage repair (regardless of whether this time occurs as arrest in G1, S or G2), prior to potential activation of apoptosis, is a critical determinant of radiosensitivity in human haematopoietic cell lines.

The mode of induction of apoptosis is dependent upon the cell type and the type and concentration of cytostatic drug used. Three different routes to the induction of apoptosis can be identified: 1. Rapid interphase apoptosis, where death occurred soon after death stimulus and in different phases of cell cycle. 2. Delayed interphase apoptosis, where death occurred following the G2 phase arrest. 3. Mitotic/delayed mitotic death, where death occurred after one or more cell division (6). To investigate whether the sensitivity of leukaemias to chemotherapeutic agents depends on the abilities of leukaemia cells to respond to therapeutic insult by inducing apoptosis, we have tested the hypothesis that the sensitivity of HL-60 cells to idarubicin is dependent on the ability of the cells to respond to the therapeutic insult by inducing apoptosis.

Original Article

DOSE DEPENDENT BIOLOGICAL EFFECTS OF IDARUBICIN IN HL-60 CELLS: ALTERATIONS OF THE CELL-CYCLE AND APOPTOSIS

Martina Mareková1, Jiřina Vávrová2, Doris Vokurková3

Charles University in Prague, Faculty of Medicine in Hradec Králové: Department of Medical Biochemistry1; Purkyně Military Medical Academy, Hradec Králové: Institute of Radiobiology and Immunology2; University Teaching Hospital in Hradec Králové: Institute of Clinical Immunology and Allergy3

Summary: TP-53 deficient cells of human leukaemia HL-60 die by massive apoptosis after treatment by high (50-100 nmol/l) doses of DNA damaging agent Idarubicin, regardless of the cell-cycle phase, in which they are affected. In contrary, after relatively low dose 10 nmol/l the cells die after cell-cycle arrest in G2 phase. The results show, that apoptosis induced by idarubicin could appear independently of the cell-cycle phase and that period in which apoptosis is observed is related to the dose of Idarubicin.

Key words: HL-60; Idarubicin; Apoptosis; G2 cell-cycle arrest

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INTRODUCTION

Acute myeloid leukaemia (AML) accounts for over 80% of all adult acute leukaemias (7) and is a characterised by a clonal expansion of immature myeloid cells in all haematopoietic tissues. Many patients progress to AML from preleukaemic myelodysplastic syndrome (MDS) or from chronic myelogenous leukaemia (CML). AMLs show varied morphologic, cytochemical, immunologic and cytogenetic characteristics and varied sensitivity to conventional chemotherapeutic regimens. Sixty percent to 70% of patients with de novo AML initially achieve complete remission. However, the majority of these patients relapse and eventually die of the disease. The first described and best characterized mechanism of resistance is mdrl gene product, Pglycoprotein. This molecule spans the cell membrane and act as an efflux pump for toxins, including chemotherapy drugs such as anthracyclines, vinca alkaloids and topoisomerase II inhibitors. The biological bases of drug resistance and relapse in AML are not understood and prognoses are still largely based on descriptive parameters. Several lines of evidence indicate that apoptosis plays roles in responses of AML patients to chemotherapy. Aldridge and Radford (1) showed that differences between human haematopoietic cell lines, in the rate of induction of apoptosis after irradiation were generally related to the functioning of cell cycle checkpoints. Whereas the rapidly dying and radiosensitive HSB-2 cell line underwent apoptosis at different points in the cell cycle, the more slowly dying cell lines showed a variety of cell cycle arrest profiles and initiated apoptosis after accumulation of cells in the G2 phase. HL-60 cells showed a markedly longer G2 arrest that correlated with their greater radioresistance. The results suggest that the total length of time available for DNA damage repair (regardless of whether this time occurs as arrest in G1, S or G2), prior to potential activation of apoptosis, is a critical determinant of radiosensitivity in human haematopoietic cell lines.

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Variable response of malignant cells to cytostatic therapeu-
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cells, second by the ability of the cytostatic to induce cell-
cycle arrest in a specified cell-cycle phase and third by the
ability to induce apoptosis. In our work we analysed cell-
cycle status of HL-60 line, duration and intensity of cell-
cycle arrest and the ability of damage repair or apoptosis
initiation after low-doses idarubicin treatment using flow-
cytometric DNA analysis.

Materials and Methods

Cell culture and culture conditions

Human leukaemia HL-60 cells were obtained from the
European Collection of Animal Cell Cultures (Porton
Down, Salisbury, UK) and were cultured in Iscove’s medi-
um (Sigma Inc.) supplemented with 20% fetal calf serum
(FCS) in a humidified incubator at 37°C and controlled 5% 
CO₂ atmosphere. The cultures were divided every 3rd
day by dilution to a concentration of 2x10⁵ cells/ml. Cell count
was performed with a haemocytometer, cell membrane in-
tegrity was determined using the Trypan blue exclusion
technique. HL-60 cells in the maximal range of 20 passages
were used for this study.

Cell treatments

Exponentially growing HL-60 cells were suspended at a
concentration of 2x10⁵ cells/ml in complete medium. 10
ml of aliquots were plated into 25 cm² flasks (Nunc) and
mixed with idarubicin (Zavedos, Pharmacia Upjohn S.p.A.
Laboratories) at desired concentrations. After 4 hours ida-
rubicin-containing medium was removed and replaced with
fresh culture medium without idarubicin. Following 6,
24, 48 and 72 hours the cells were counted and cell viabili-
ty determined with the Trypan blue exclusion assay.

Cell morphology

For calculation of the percentage of cells showing mor-
phology of apoptosis, aliquots were removed from control
and drug-treated cell cultures at various times of incubation
and usually 400 cells were counted on Diff-Quik stained cyto-
spin preparations. Apoptotic cells were identified by the condensed and fragmented state of their
nuclei and focal protrusions of the cell surface.

Cell surface markers and cell size analysis

Flow cytometry was used for cell surface antigen analy-
sis and for apoptosis monitoring. Cells were washed twice
with PBS containing 5% FCS and mixed with idarubicin (Zavedov, Pharmacia Upjohn S.p.A.
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Cell cycle analysis

Following 6, 24, 48 and 72 hours of incubation, the
cells were washed with cold PBS, fixed by 70% ethanol and
stained with propidium iodide (PI) in Vindelov’s solution
for 30 minutes at 37°C. Fluorescence (DNA content) was
measured with Coulter Electronic, Hialeah, FL, USA appa-
ratus. A minimum of 10 000 cells analysed in each sample
served to determine the percentage of cells in each phase of
cell cycle, using MultiCycle AV software. Three indepen-
dent experiments were performed.

Results

Cell growth and viability

Fig. 1 shows the effects of idarubicin on the proliferati-
ve rate of HL-60 cell line. Cultivation with 5 nmol/l idaru-
bicin induced high inhibition of the rate of HL-60 cell
growth. The decrease of the proliferative rate observed in
HL-60 cells after addition of higher concentration of ida-
rubicin was observed. After 48 hours all cells with 100 and
50 nmol/l idarubicin were dead.

Morphologic changes

HL-60 cells were incubated in the presence 5, 10, 20,
50 and 100 nmol/l idarubicin for 72 hours. After 1, 6, 24,
48 and 72 hours cell morphology was examined on Diff-
Quik stained cyto spin preparations. Morphologic evidence
of apoptosis was found in cells treated with idarubicin.
After addition of idarubicin dose dependent increase in
the proportion of apoptotic cells was detected in cultures
exposed to 5-100 nmol/l idarubicin. The maximal percen-
tage of apoptotic cells was observed in cultures incubated
for 6 or 24 hours treated with 100 or 50 nmol/l idarubicin
respectively (Fig. 2). During in vitro studies, where apa-
ptotic cells cannot be removed by fagocytosis, secondary
carcinosis can be observed in later intervals (24 to 72 hours
in Fig.2).

Analysis of cell-cycle and sub-diploid DNA content

We assessed DNA cleavage in the afore mentioned 5
and 100nmol/l idarubicin-treated tumour cells. We have
observed that after 6 hours of incubation most of the live
idarubicin-treated cells were in S phase of cell cycle (62% or
71%, respectively), after 24 hours most of them moved to
G2 phase (61% or 75%) and after 48 hours the percentage
of cells in various cellcycle phases was comparable to con-
trol untreated cells (Fig.3). Results of one representative ex-
periment with idarubicin concentration 5 nmol/l are shown
in Fig. 4.

Fig. 1: Kinetics of idarubicin effect on the proliferative rate
of HL-60 cell line. HL-60 cells were exposed to various ida-
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cultivated in idarubicin-free medium. Number of viable
cells was determined by Trypan blue staining.

Fig. 2: Time course of apoptosis in HL-60 cells exposed to
idarubicin as determined by cell morphology examined on
Diff-Quik stained cyto spin preparations. Data represent
medium values from 3 independent experiments.

Fig. 3: Flow cytometric analysis of DNA content and cell-
cycle after treatment with 5 and 10 nmol/l idarubicin.

Fig. 4: Flow cytometric analysis of DNA content and cell-
cycle after treatment of HL-60 cells with 5 nmol/l idarubi-
cin. Apoptotic cells are identified as cells with subdiploid
DNA content (lower DNA content than cells in G2/G0 pha-
se). i.e. subG1 peak. Representative results for single expe-
riemnt are shown.

Fig. 5: Histograms for cell number versus APO2.7-PE fluorescein intensity of unprocessed HL-60 cells after treatment
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Flow cytometry was used for cell surface antigen analy-
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and incubated with mAb APO2.7 (clone 2.7 A6A3) (obtained from
Immunotech) for 30 min at 4°C. Flow cytometric analysis was performed on a Coulter
Epics XL flow cytometer. A minimum of 10 000 cells was
collected for each sample in a list mode format. List
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Fig. 3 shows the effects of idarubicin on the cell cycle
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Fig. 3: Flow cytometric analysis of DNA content and cell-
cycle after treatment with 5 and 10 nmol/l idarubicin.
Flow cytometric detection of apoptotic cells using monoclonal antibody APO2.7

Since 7A6 antigen is selectively expressed on the mitochondrial membrane in cells undergoing apoptosis, we attempted to detect apoptotic cells using APO2.7 monoclonal antibody after 5 and 10nmol/l idarubicin treatment of HL-60 cells. As shown in Fig.5 in unirradiated cells APO2.7 antibody staining significantly increased from 6% at 4h to 65% at 72h after treatment with 10nmol/l idarubicin, while increased only slightly after treatment with 5nmol/l idarubicin (from 6% at 4h to 16% at 72h) (Fig. 6).

Discussion and Conclusions

HL-60 line has amplified myc and activated ras oncogene, it is p53 negative and does not contain the characterisitic t(15:17) translocation seen in acute promyelocytic leukaemia (3, 4). HL-60 cells were negative for expression of CD34 and AC133 antigens (0.6/0.7%), the antigens usually used for separation of haematopoietic progenitors from mobilised peripheral blood patients for autologous transplantation. HL-60 cells expressed high levels of CD15 (93%) and CD33 (84%) antigens, 82% cells were CD15+/CD33+ (5). Haematopoietic progenitor cells giving rise to monocytic and granulocytic lineages express numerous surface antigens to varying degrees depending on the cell cycle arrest. CD33 antigen is expressed prior to myeloid commitment and CD15 is expressed at later stages in myelomonocytic development. Hofmanová et al. (3) described that 90% HL-60 cells were promyelocytes and 8% myelocytes with no expression CD14/CD11b antigens.

We show in this study that relatively high doses of idarubicin (50-100 nmol/l) induce apoptosis soon after treatment (6 hours). We presume that the cells die by rapid interphase apoptosis, where the apoptosis is triggered in all phases of cell cycle. Some studies (8) suggest that apoptosis induction and G1 or G2 cell cycle arrest are two separate phenomena in Jurkat cells (T-cell line, mutated gene TP53, undetectable levels of TP53 protein). It has been shown (in accordance with thesis that cells with mutated TP53 are radiosensitive) that apoptosis occurs in these cells during 24 hours after irradiation by high doses of ionising radiation(10-20 Gy). Regardless of the cell cycle phase 20% of apoptotic cells have been detected 6 hours after irradiation dose 20 Gy. Apoptosis was lower when early G1 or G2 subpopulation has been irradiated in comparison to other cell cycle phases. It seems that after irradiation of cells in G1 phase apoptosis occurs 2 hours later in comparison with cells in other cell cycle phases. However, 24 hours following irradiation by 20 Gy all cells were apoptotic regardless of the cell-cycle phase, in which they were irradiated.

Relatively lower doses of idarubicin (5-10 nmol/l) first inhibit proliferation of the cells and induce changes in cell cycle. We have observed that after 6 hours of incubation most of the live cells accumulate in S phase of cell-cycle, after 24 hours we observed arrest in G1 phase. As 48h following 10 nmol/l idarubicin treatment we observed significant apoptosis and the cells did not proliferate during 72 hours. Lower dose (5 nmol/l) induces only small percentage of apoptosis after cell-cycle arrest in G1 phase and the cells slightly proliferate during 72 hours following idarubicin treatment. We suppose that after 10 nmol/l the cells die by delayed interphase apoptosis, which typically occurs after cell-cycle arrest in G2 phase. Similar results have been reported after irradiation of HL-60 cells by 5-Gy, where the apoptosis occurs following cell-cycle arrest in G2 phase 36-48 hours after irradiation (9). Cellcycle arrest in G2/M phase has been observed in Jurkat cells (8) following irradiation by relatively low dose 2 Gy, regardless of cell-cycle phase, in which they were irradiated. Apoptotic cells were cumulated 25-50 hours after irradiation. It is interesting that apoptosis occurred sooner in population of cells irradiated in G2 phase in comparison to other cell-cycle phases.

It can be concluded that apoptosis induced by low doses of idarubicin in TP-53 negative cells HL-60 as well as apoptosis induced by ionising radiation in dose 2-10 Gy in various TP-53 negative haematopoietic cell lines (Jurkat, HL-60) occurs following G1 cell-cycle arrest. Apoptosis was observed at HL-60 cells as soon as 6 hour after treatment with high idarubicin concentrations (50-100 nmol/l), similarly to Jurkat cells irradiated by supralethal doses (10-20 Gy), which also underwent apoptosis after 6 hours. Syljuasen and McBridge (8) proved that Jurkat cells treated by ionizing radiation could undergo apoptosis independently on cell-cycle arrest and that period in which apoptosis is observed is related to the dose of radiation. Similar results show from experiments on idarubicin influence on HL-60 cells.

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