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Apoptosis, p53 and DNA Damage in Human Lymphoblast Cells: Relation to Cell Death after Radiation

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ABSTRACT

Purpose: To analyse the importance of both clonogenic and apoptotic death of lymphoblast cells after ionising radiation and their relation to p53 expression and radiosensitivity.

Materials and methods: Two lymphoblastoid cell lines differing in the expression of p53 ($p53^{wt}$ and $p53^{mut}$) were compared as regards (a) intrinsic radiosensitivity by clonogenic assay, (b) initial and residual DNA double strand breaks (DSBs) as determined by constant field gel electrophoresis, and (c) induction of apoptosis as assayed by agarose gel electrophoresis.

Results: As expected the $p53^{wt}$ cell line was 4-fold more radiosensitive than the $p53^{mut}$ line as measured by the surviving fraction after 2 Gy (SF_2). The initial DSB induction did not differ in the two lines. The fast repair component during the first post-radiation hour did not differ in the two lines. This was followed by a slow repair component which appeared earlier in the $p53^{wt}$ cell line as indicated by the values at two hours. The 2h-interval was chosen due to a renewed increase in the fraction of DNA release that coincided with induction of apoptosis. As expected apoptosis appeared earlier in $p53^{wt}$ line and was greater in magnitude.

Conclusion: These results support the concept that intrinsic radiosensitivity in $p53^{wt}$ line is not related to induction of DSBs but apparently correlates with the slow repair component and the magnitude of the apoptotic response.

Key Words: *p53 - Apoptosis - Radiosensitivity - DSB - Lymphoblast cells.*

INTRODUCTION

The predominant mechanism by which radiation kills mammalian cells is believed to be the reproductive (clonogenic) mode of cell kill principally due to DNA double-strand breaks. Although most radiation-induced DNA breaks are rapidly repaired, residual or incorrectly repaired breaks lead to genetic instability, increased mutation frequency and chromosomal aberrations **[3, 7, 33]** which may lead to cell death usually after a number of mitotic cycles **[11,34]**.

Apoptosis can be triggered by a variety of stimuli such as ionising or UV radiation **[17, 30, 35]**, drugs and hyperthermia **[2, 10, 18]** and some hormones **[41]**. These agents activate endogenous nucleases that cleave chromatin in a non-random manner resulting in a series of DNA fragments which are integer multiples of 180-200 base pairs **[40]**. Published data concerning the extent of radiation-induced apoptosis versus clonogenic cell death showed that after single dose that result in 90% clonogenic cell kill, up to 50% could be attributed to apoptosis. Modulation of the processes involved in apoptosis may therefore result in new modalities for cancer therapy. In addition, measurement of the extent of apoptosis during treatment with apoptogenic agents may help in monitoring the response of certain types of tumours to treatment **[9]**.

P53 is the most commonly mutated gene in human tumours with a large impact on cellular biology and response to radiation. After radiation, p53 controls cell-cycle progression, DNA repair and regulates apoptosis mainly in hematopoetic tissue. Mutations of p53 alter these functions which may be responsible for changed cellular and tumour response to radiation treatment. After ionising radiation, cells may undergo p53-dependent apoptosis. The central domain of p53 serves as transcription factor for the bax promotor **[19]**. Bax is one of the major apoptosis genes while its antagonist, the apoptosis protector bcl2, is down-regulated by p53 **[20]**. P53 also regulates other apoptosis

pathways through PIGs (p53 inducible genes). Both p53-dependent pathways might commonly trigger a caspase cascade leading to apoptotic degradation.

Consequences of direct and indirect effects of ionising radiation on DNA are base damage, DNA-protein crosslinks and DNA single and double-strand breaks (DSB). Current evidences support the notion that unrepaired or misrepaired strand breaks, particularly double strand breaks, have the most catastrophic consequences for the cell in terms of loss of reproductive integrity [13,24]. Many studies attributed differences in radiosensitivity between different tumour cells to initial induction of DNA DSB [13,14,21,23,24,25,36] while others failed to confirm this correlation [6,8,12,29,32,37,38]. On the other hand repair of DSB was reported by some investigators to correlate with cell death after ionising radiation in both normal and tumour cells [27,28,39,44]. This type of correlation was not found in some other studies [26,32]. Up till now it is not clear whether the extent of induction and repair of DSBs have an impact on extent and rate of apoptosis.

The human B lymphoblast cell lines WTK1 and TK6 are both heterozygous for the autosomal thymidine kinase locus [4,31] and are derived from the same cell line (WIL2) originally isolated from a male human spleen. These two cell lines have very similar cytological and growth characteristics but differ in their p53 status. WTK1 was shown by many investigators [15,42] to overexpress a mutant form of p53 (methionine to isoleucine substitution at codon 237) whereas TK6 has a wild-type p53. Chinese hamster ovary cells (CHO) which was previously shown to resist apoptosis even after exposure to high radiation doses was used in the present study as control.

The main aim of this study was to analyse the importance of both clonogenic (resulting from DNA double-strand breaks) and apoptotic death of lymphoblast cells after ionising radiation and their relation to p53 expression and radiosensitivity.

MATERIALS AND METHODS

Cell lines and culture conditions:

The two human lymphoblastoid cell lines, TK6 (ATCC CRL 8015) and WTK1 (ATCC CRL 8155), were grown in suspension cultures in growth medium consisting of RPMI 1640 medium (Gibco BRL) supplemented with 10% heat inactivated fetal calf serum (FCS), penicillin (100U/ml) and streptomycin (100µg/ml). CHO cells, obtained from ATCC, were grown as monolayer cultures in alpha minimal essential medium (Sigma) containing 10% fetal calf serum, penicillin (100U/ml) and streptomycin (100µg/ml). All cells were incubated at 37°c in 5% CO_2 and 100% humidity.

Clonogenic cell survival:

Cell survival was assessed by colony formation assay. Exponentially growing cell suspension in case of TK6 and WTK1 or monolayer in case of CHO cells were irradiated at room temprature using a Philips X-ray machine operating at 220 kVp, 20 mA with 0.5 mm Cu filter at a dose rate of 1Gy/min. Irradiated TK6 and WTK1 cells were directly seeded into Petri-dishes (5 cm) whereas CHO cells were firstly trypsinised and seeded into Petri-dishes at appropriate cell concentration. Two weeks after plating, the cells were fixed, stained with crystal violet and colonies of more than 50 cells were counted. For each cell line three separate experiments with three replicates were performed. The data were fitted by nonlinear regression using the linear-quadratic equation:

$$- \ln S = \alpha D + \beta D^2$$

Where S is the surviving fraction, D is the x-ray dose, a and b are the initial and final slope of the survival curve respectively

Induction of DSB by graded-field gel electrophoresis (GFGE)

Induction of DSB was meaured by GFGE [6]. Briefly, 6x10^6 cells/ml cell suspension was mixed with an equal volume of 1.6% low melting point (Bio-Rad) agarose solution at 37°C. This mixture was pipetted into 180µl plug moulds and left to solidify on ice. The agarose cell plugs were irradiated on ice to prevent DNA repair during irradiation and were transferred after irradiation to lysis buffer (0.4 M EDTA, 2% N-lauryl sarcosine, and 1mg/ml proteinase k). Lysis was started on ice for 30 minutes and continued at 37°c overnight. The plugs were washed three times with tris-EDTA buffer at room temprature and sliced into pieces containing about 10^5 cells, which were inserted into a 14x20 cm 0.8% agarose gel in 0.5 x TBE buffer (45mM Tris base, 45 mM boric acid, 2

mM EDTA). GFGE was performed in a conventional apparatus (Subcell, Bio-Rad, Germany). Running conditions were 0.6 V/cm for 30 h followed by 1.5 V/cm for 6 h in 0.5x TBE buffer. After completion of electrophoresis the gel was stained overnight in 0.5 µg/ml ethidium bromide solution, destained overnight in distelled water and a video picture was taken under UV transillumination. The fraction of DNA released (FDR) was quantified using a CCD camera (Sony XC-75CE) equiped with an image analysis system (Optimas, Silverspring, MD, USA).

Constant-field gel electrophoresis for measurement of DSB repair and apoptosis:

Cells were irradiated either as suspension (in case of TK6 and WTK1) or as monolayer (in case of CHO) and incubated at 37°c. After different repair time intervals, cells were collected and treated as described above. The fraction of DNA released after each repair time was determined by means of constant-field gel electrophoresis (0.6 V/cm for 30 h).

Statistical analysis:

Each experiment was repeated three times and the data were given as a mean with its standard error (SEM). Statistical analysis, data fitting and graphics were performed by means of the GraphPad prism computer program (GraphPad Software, San Diego, USA).

RESULTS

Cell survival:

Cellular radiosensitivity of the three cell lines is shown in figure [1]. WTK1 cells showed an enhanced survival following irradiation as compared to TK6 cells whereas CHO cells were the most radioresistant. The linear-quadratic equation was fitted to the data in figure 1 and the parameters obtained are listed in table (1).

Induction of DNA double-strand breaks:

Fig. 2 shows separation of radiation induced DNA fragments in TK6 cell line by the graded-field gel electrophoresis, GFGE. Since two voltages were used, two bands containing DNA fragments with different molecular weights were obtained. The first band contained fragments of 5.5-11 Mbp, while the second band assembled all fragments with a molecular weight below 5.5 Mbp.

As shown in figures 2 and 3, the fraction of DNA released (FDR) to band 1 increased gradually with the radiation dose reaching a maximum at a specific dose and declined thereafter, while FDR to band 2 increased continuously to reach an apparent plateau at about 75-80% for the three cell lines. High radiation doses almost completely reduced large fragments contained in band 1 to smaller sizes and hence all DNA released was collected in band 2. The three DSB induction curves in figure 3 have similar shapes. In addition, band 1 reached its maximum at similar radiation doses in the three cell lines (28, 30.5 and 25.8 Gy for TK6, WTK1 and CHO cells respectively). As outlined previously [6], variation in the radiation dose at which band 1 reached maximum solely depends on the number of DSB induced. Thus, no difference in the number of induced DSB was shown between both lymphoblast cell lines (7.7 and 8.1 x 10^{-12} DSB/Gy/Da for TK6 and WTK1 cells respectively).

CHO cells were measured in parallel to validate earlier results [6]. The profile of band 1 and 2 perfectly matched the previous observation, however, the number of induced DSB is now calculated to be slightly smaller than before (9.2 ± 0.6 versus 11.5 ± 0.2 x10^{-12} DSB/Da/Gy).

Rejoining of DSB and apoptosis:

Rejoining of DSB in TK6 and WTK1 cells irradiated to a dose of 60 Gy and incubated for different repair time intervals was measured by means of constant-field gel electrophoresis. As shown in fig. (4-A), FDR decreased in CHO cells with increase in repair time (due to repair of DSB) along the whole repair time. FDR from irradiated WTK1 cells decreased also with the repair time up to 8 h after which unexpected increase in the FDR with time was observed (Fig. 4-B). On the other hand, the extent of decrease in FDR with the repair time in irradiated TK6 cells (Fig. 4-C) was much lower than in WTK1 cells and increase in FDR during repair incubation was more pronounced and appeared earlier (after 4h) than in WTK1.

Fig. (5) shows the kinetic of DSB repair in both lymphoblast cell lines. Both cell lines repaired most of DSB rapidly and at the same rate during the first hour. This rapid repair phase was followed by a slow phase during which repair of DSB in TK6 (with wild type p53) was

slower and less efficient than in WTK1 cells (with mutant p53). This slow phase persisted up to 4h in both cell lines and was followed in WTK1 cells by a plateau phase persisting up to 8 h during which no more DSB was repaired. In TK6 cells, the slow phase was not followed by a plataeu phase but a rapid increase in FDR was observed (consistent with apoptotic DNA degradation) where the amount of DNA damage exceeded after 16h repair the initial damage at 0 repair time. This increase in DNA damage was also seen in WTK1 cells but after 8 h and was lower than those observed in TK6 cells (did not exceed the initial DNA damage).

Repair of DSB in the control CHO cells (Fig. 5) can be characterized from that of both lymphoblast cells by three main differences. First, it was more rapid and efficient especially during the first hour where about 80% of the initial DSBs were repaired. Second, the plateau phase of repair was attained after 4h and persisted up to 24h. Finally no apoptotic DNA degradation was shown and the number of DSB after 24h repair attained the value of control unirradiated cells.

Table (1): Radiosensitivity parameters.

Cell line	α^a	β^a	SF_2^b
CHO	0.1219 ± 0.04	0.061 ± 0.003	0.615 ± 0.03
TK6	1.348 ± 0.221	0.0124 ± 0.008	0.072 ± 0.002
WTK1	0.4749 ± 0.06	0.0875 ± 0.005	0.274 ± 0.015

[a] Linear and quadratic terms of the survival curve determined for data shown in figure 1 using nonlinear regression.

[b] Surviving fraction calculated for an x-ray dose of 2 Gy.

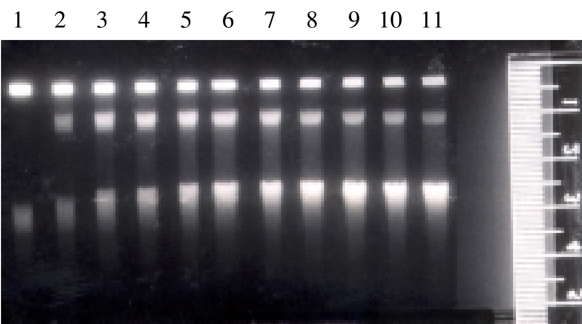

Fig. (2): Graded-field DNA electrophoresis. TK6 cells were embedded in agarose, irradiated at 4°c and subjected to GFGE. The EB-stained gel shows the wells (upper), the band fo DNA migrating at 0.6 V/cm (middle) and the band of DNA migrating at 1.5V/cm (lower). Lane 1 contains control unirradiated cells, lanes 2-11 contain samples irradiated with radiation doses 10-140 Gy.

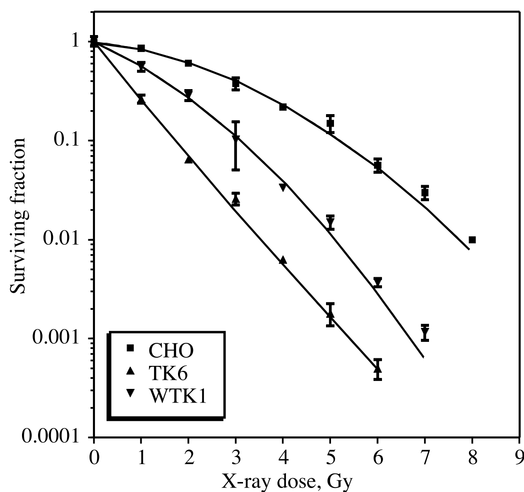

Fig. (1): Cell survival curves measured for three cell lines. Cells were irradiated, trypsinised and immediately plated for colony assay. The data were fitted by a linear quadratic equation and the parameters are listed in table 1.

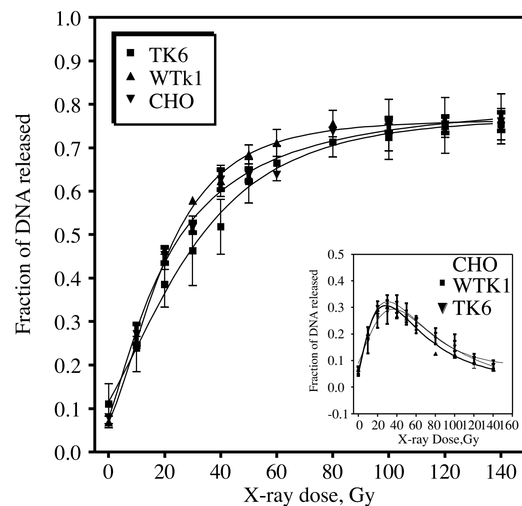

Fig. (3): Induction of DNA dsb in TK6, WTK1 and CHO cells. Cells were irradiated on ice, lysed and DNA fragments were isolated by GFGE technique. Inset shows fraction of DNA released to band 1in the same three cell lines.

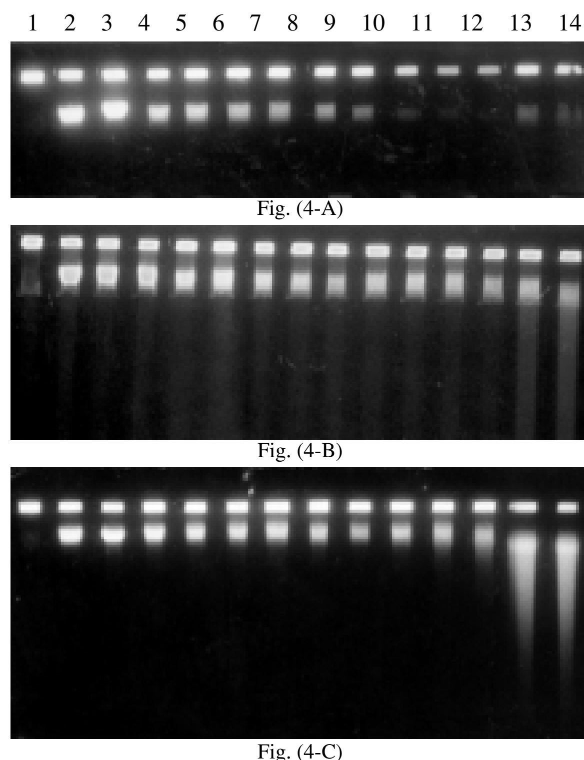

Fig. (4): kinetics of dsb repair in (A) CHO, (B) WTK1 and (C) TK6 cells. Cells were irradiated on ice with 60 Gy, incubated at 37°c and after different repair time intervals samples were lysed, and FDR was determined using CFGE (0.6 v/cm for 30 h). The figure shows appearance of apoptotic DNA degradation in WTK1 and TK6 cells and its absence in control CHO cells.

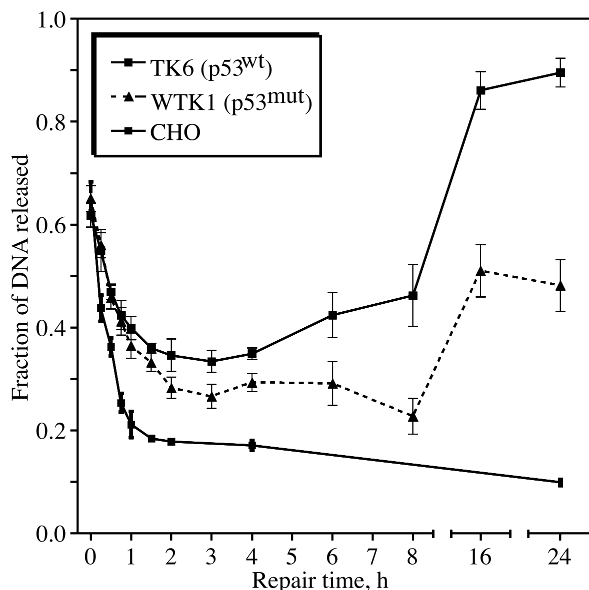

Fig. (5): kinetics of dsb repair in TK6, WTK1and CHO cells. Cells were irradiated as monolayers on ice with 60 Gy. Repair was started directly after irradiation by incubating the cells in fresh medium at 37°c and after different repair time intervals, samples were lysed and separated by CFGE (0.6 V/cm for 30h). The figure shows emergence of apoptotic DNA degradation in both lymphoblast cell lines at different times and rates and its absence in CHO cells.

DISCUSSION

Induction of DNA double-strand breaks was reported in several previous studies to trigger apoptosis in many cell types [22]. It was therefore, justified to investigate whether differences in the initial number of radiation-induced DSBs can explain differences in the extent and rate of apoptosis in the two lymphoblast cell lines differing in the status of p53. Another aim of this study was to test the hypothesis that differences in radiosensitivity between two lymphoblast cell lines can largely be attributed to differences in the p53 status, rate and extent of apoptosis and efficiency of DSB repair. No differences between both lymphoblast cell lines were found as regards the number of initially induced DSBs. This finding explains, therefore, that the number of initially induced DSBs is not a contributing factor which controls the extent and rate of apoptosis in different cell types. It seems that only very few number of DSBs at specific sites of the genome are sufficient to trigger apoptosis and once these sites are hit, apoptosis is initiated and more additional DSBs produce therefore no effect on the rate and extent of apoptosis. But the higher the number of DSBs the higher the probability of occurence of DSBs at these triggering sites.

We considered the possibility that the number of residual (non-reparable) DSBs may have an impact on the rate and extent of apoptosis in both lymphoblast cell lines. This principle seemed to be correct to a great extent because TK6 cells which showed a higher extent and a more rapid onset of apoptosis showed more residual DSBs than the WTK1 cells which had a lower extent and a slower rate of apoptosis (Fig. 5). This may indicate that the number of residual (non reparable) DSBs rather than the number of initially induced DSBs may determine the extent and rapidity by which apoptosis occure in cells.

This finding was confirmed by our results from the control cells used in this study, CHO cells, where the level of residual DSBs in these cells was very low and 8 h after irradiation the number of residual DSBs was similar to control unirradiated cells (Fig. 5). CHO cells are known to have no apoptotic activity which confirms the suggestion that number of residual DSBs may have an impact on the rate and extent of apoptosis. Although induction of apoptosis after DNA damage has been described by

different authors to depend mainly on wild-type p53 [1,5,16,43], results of this study showed that both lymphoblast cell lines with different p53 status were capable of expressing apoptosis although at different rates and extent. This may indicate that p53 mutant cells can use p53-independent pathways to excute apoptosis or that p53 mutation in WTK1 cells was not at the p53 site responsible for apoptosis. These results agree with the results of Xia et al. [42] who found differences only in the rate but not the extent of apoptosis between the same lymphoblast cell lines. However, Xia et al. followed the cells for up to 3 days after irradiation and reported that the extent of apoptosis in both cell lines was similar only 2 days after irradiation wheras in the present study cells were followed only for 24 h after irradiation.

Resistance of many cells to radiation was ascribed by many investigators to the kinetic and efficiency of DSB repair [28,39,44]. Results of the present study are in agreement with this finding where the more resistant lymphoblast cell line (WTK1) showed ,before onset of apoptosis, more efficient DSB repair than the more sensitive line (TK6). On the same line, CHO cells which were used in this study as control and were 2.2 and 8.5 times more radioresistant than WTK1 and TK6 cells respectively showed more rapid and efficient DSB repair than both lymphoblast cell lines.

In conclusion, differences in radiosensitivity between both lymphoblast cell lines can not be attributed to differences in the number of initially radiation-induced DSBs. The number of initially induced DSBs does not depend on of p53 status nor can explain differences in apoptosis between both lymphoblast cell lines. Differences in DSB repair efficiency may contribute to a great extent to differences in the extent and rate of apoptosis which in turn may partly explain the differences in radiosensitivity. p53 is not the sole way of inducing apoptosis in these lymphoblast cell lines and finally both clonogenic (resulting from residusl DSBs and chromosomal abberations) and apoptotic cell death contirbute to cell death after irradiation of lymphoblast cells.

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