

Caspase Dependent Apoptosis is Only Inhibited on Γ Irradiation of Cells Conditioned by Repetitive Oxidative Stress

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Abstract: *V79^C cells were derived from V79 cell line by through chronic oxidative stress that was found to be radio-resistant. These cells had demonstrated transformation-like stable changes and could be used as model system to study oxidant induced carcinogenesis. Our objective was to understand mechanism of radiation-resistance in these cells. Apoptotic cell death was inhibited in these cells as visualized microscopically by Hoechst staining and nucleosomal ladder formation in agarose gel. Release of cytochrome c in cytoplasm and Apoptosis Inducing Factor in the mitochondrial and nuclear fraction of cells were determined by Western blotting. The caspase 9 and caspase 3 activities in these cells were estimated from fluorimetric assays. These results revealed that the radiation resistance was due to inhibition of caspase-dependent apoptotic death pathways although Apoptosis Inducing Factor mediated pathway remained unaffected. These findings may aid in understanding the mechanism of radiation resistance in tumors arising from oxidative stress.*

Keywords: γ rays, Cytochrome c, Caspases, Apoptosis inducing factor

1. Introduction

Oxidative stress is known to induce several genes that include transcription factors, cell cycle regulators, heat shock proteins, those involved in DNA damage tolerance and repair, recombination, apoptosis and most importantly the antioxidant enzymes [1]-[3]. This often leads to aberrant cell growth or cancer [4]. In fact the role of oxidative damage in the etiology of cancer is well established [5], [6]. Yet, most effects of oxidative stress have been studied with single exposure to a high dose of oxidant. However, physiologically it is more relevant to see the effects of chronic low dose exposures.

In this endeavor, we have derived a cell strain V79^C from Chinese hamster lung fibroblast V79 cell line through repetitive low dose treatment with H₂O₂. Cells through their intrinsic ability for adaptation achieve recovery from chronic stress. These cells acquired several traits of transformation that includes higher aneuploidy, better tolerance to serum starvation and significantly higher antioxidant defense compared to its parental V79 cells [7], [8]. Up-regulation of antioxidant defense has often been correlated to apoptotic process [9]-[11]. Also, many tumor cell lines are known to be radio-resistant due to inhibition of apoptosis [12]. These conditioned V79^C cells can be used as models for oxidant-induced carcinogenesis, to study apoptosis in these cells.

The role of apoptotic cell death in the resistance to cell killing by γ rays in V79^C cells has been studied. Apoptosis was scored by staining nuclei with Hoechst dye and also from nucleosomal ladder formation in DNA gel electrophoresis. Mitochondria play a pivotal role in regulating apoptosis. It harbors several pro-apoptotic proteins that are released to the cytoplasm upon appropriate signals [13]. One of the mechanisms of apoptosis involves release of cytochrome c from the mitochondria [14]. The apoptotic cell present specific biochemical alterations including activation of specific proteases called caspases. Caspase activation leads to action of specific nucleases that

result in the characteristic pattern of nuclear fragmentation [15], [16]. Release of cytochrome c from mitochondria and activities of caspase 9 and caspase 3 have also been determined in these cells to investigate the involvement of caspase-dependent pathways of cell death. Another critical molecule is the apoptotic inducing factor (AIF), which causes chromatin condensation and DNA fragmentation, but acts through caspase-independent pathway [17]. The AIF released in both types of cells after γ irradiation was estimated to consider the role of AIF-dependent apoptotic pathway in these conditioned cells.

2. Materials and Methods

2.1. Chemicals and antibodies

Eagle's minimal essential medium (MEM) was obtained from Hi Media, India and agarose, acrylamide, bis-acrylamide, Triton X-100, Hoechst 33258 dye, RNase A, Tween 20, β -mercaptoethanol, anti- β -actin antibody and anti-AIF antibody were obtained from Sigma Chemicals (USA), secondary antibody (anti-mouse) and horseradish peroxidase conjugated were from Santa Cruz Biotechnology (USA) and proteinase K, was from Life Technologies (USA). Anti-cytochrome c antibody was from Pharmingen (Becton Dickinson, USA), and secondary antibody (anti-mouse and anti-rabbit) from Santa Cruz Biotechnology (USA), N-hybrid membrane from Amersham Biosciences, X-ray film, developer and fixer were from Kodak. Other molecular biology grade fine chemicals were procured locally.

2.2. Cell Culture

Chinese hamster V79 cells and its derived V79^C cells were utilized for these studies. Growth conditions for cells were as described earlier [18], [19]. Briefly, the cells were grown in MEM supplemented with 10% dialyzed goat serum and

antibiotics (penicillin and streptomycin) at 37°C in a humidified 5% CO₂ atmosphere.

2.3. Conditioning Cells by Chronic H₂O₂ Treatment

Conditioning of cells with chronic exposure to oxidant was as described earlier [20]. Briefly, population of V79 cells was exposed to a nontoxic dose of H₂O₂ (30 µM) for 1 hour, each day for 5 days/week. After each treatment the cells were washed in serum free medium and allowed to grow in fresh medium. In total the cells received 60 such conditioning treatments over a span of 12 weeks. During this period the cells were maintained in the exponential phase of growth by splitting them 2-3 times a week. The cells conditioned in this fashion were designated as V79^C cells. Batches of these cells were also kept frozen in liquid nitrogen after completion of the conditioning treatment. Experiments were done to compare the response of these conditioned cells with its parental V79 cells. The parental V79 cells were maintained under identical growth conditions and these cells were also maintained at exponential growth phase by splitting them at the same frequency.

2.4. γ -Irradiation

Exponentially growing V79 and V79^C cells were exposed to γ rays from a ⁶⁰Co source having dose rate 1.65 Gy/min. A dose of 6 Gy was chosen for all experiments where the surviving fraction of V79 cells was ~ 0.3. After γ irradiation, cells were washed in serum free medium and fresh growth medium was added to each petridish and cells were further incubated till processing as needed in each experiment.

2.5. Apoptosis assay by Hoechst staining

The morphological changes of the cell nucleus during apoptosis were observed microscopically after staining the cells with Hoechst as described by Giri et al [21]. Two petridishes each of unirradiated and γ irradiated V79 and V79^C cells, cultured on cover-slips were taken for Hoechst staining. As evidences of apoptosis were visualized 24 hours after treatment, both the cells were processed 24 hours after γ irradiation. Cells were fixed in Acetomethanol (1:1; acetone: methanol) for 1 hour at 4°C. Then cells were incubated in 1 mM Hoechst in PBS in dark at room temperature for 4 minutes, excess stain was washed off with PBS and cells were observed under fluorescent microscope (Carl Zeiss, Axiokop 2 plus with appropriate attachments) using UV filter. At least 200 cells each were scored for apoptosis at 40X magnification. Fraction of cells with apoptotic nuclei was calculated by taking the ratio of number of cells with apoptotic nuclei to total number of cells counted. The average and standard deviations of three independent experiments were plotted.

2.6. Apoptosis Detected by Nucleosomal Ladder Formation

Apoptosis was detected in DNA gel through nucleosomal ladder formation, a hallmark of apoptosis, as described earlier [22]. The method was essentially as described by Herrmann et al with slight modifications [23]. Exponentially growing V79 and V79^C cells after exposure to γ ray was

incubated in fresh medium. The cells were trypsinized and suspended in PBS 24 hours after treatment. 5 × 10⁶ cells were taken in each case. The cell pellet obtained on centrifugation was lysed in buffer containing 50 mM Tris, 20 mM EDTA, 1% Nonidet P40 for 10 minutes at room temperature. After lysis it was centrifuged for 10 minutes at 1600 g. SDS was added to the supernatant at a final concentration of 1%, and then treated with RNase A (final concentration 0.1 mg/ml) overnight at 56°C. This was followed by Proteinase K (final concentration 0.1 mg/ml) treatment for 6 hours at 56°C. Next, 0.5 volume of 10 M ammonium acetate was added, along with 2.5 volumes of absolute ethanol and allowed to stand overnight at -20°C. The precipitated DNA was run in 1.5% agarose gel and apoptotic ladder was visualized under UV illumination after staining with ethidium bromide (0.5 µg/ml) and was photographed. Untreated cells were processed in the same way and used as control. This experiment was done twice and the result shown is from one such experiments.

2.7. Fluorometric Determination of Caspase 9

Assay of caspase 9 activity was performed according to the protocol given by the manufacturers (BD biosciences, Pharmingen, USA). Control cells and cells exposed to γ rays were assayed for caspase 9 activities. In γ irradiated cells, the assay was done 18 hours after exposure. 10⁶ cells were taken and centrifuged at 400 g for 5 minutes. Trypsinized cells were washed twice with ice cold PBS and lysed with the supplied lysis buffer. The lysate was cleared by centrifugation at 18000 g for 5 minutes at 4°C. 2X reaction buffer/reaction mix (10 µl 1 M DTT/1 ml 2X reaction buffer) were freshly prepared. 50 µl of this mixture was added to 50 µl of the supernatant and incubated on ice for 30 minutes and 5 µl of caspase 9-substrate (LEHD-AMC; 250 µM final concentration) was added to each tube and incubated at 37°C for 1 hour. The fluorescence from the liberated AMC was measured in spectrofluorometer with excitation at 380 nm and emission at 460 nm. The results shown are the mean of two sets of experiments.

2.8. Fluorometric Determination of Caspase 3

Assay of caspase 3 activity was performed according to the protocol of the manufacturer (PharMingen (Becton Dickinson), USA). 18 hours after γ -irradiation both V79 and V79^C cells were trypsinized and counted. 10⁶ cells were taken and centrifuged at 400 g for 5 minutes. The pellet was washed twice with ice cold PBS and then lysed using the supplied lysis buffer. The lysate was cleared by centrifugation at 18000 g for 5 minutes at 4°C. The protein concentration of the supernatant was determined and made to 3 µg/µl. An aliquot of 300 µg of protein was then added to 1 ml of 1X HEPES buffer (reaction buffer) (2X HEPES buffer: 40 mM HEPES pH 7.5; 20 % glycerol; 4 mM DTT. It was diluted to 1X with sterile dH₂O and stored at room temperature prior to use). 10 µl of Ac-DEVD-AMC, the substrate for caspase 3 was added to each reaction mix and allowed to incubate for 1 hour at 37°C. The fluorescence of liberated AMC was measured in a spectrofluorometer (Perkin-Elmer, LS50B, U.K.) by excitation at 380 nm and emission at 437 nm. The assay was repeated thrice.

2.9. Western blot analysis for cytochrome c and apoptotic inducing factor (AIF)

Both unirradiated control and γ irradiated V79 and V79^C cells were used for Western blot analysis. 18 hours after treatment cells were trypsinised and washed twice with PBS for separation of nuclear, cytoplasmic and mitochondrial extracts according to Martin et al [24]. This was further used for immunoblot detection for cytochrome *c* and AIF (apoptotic inducing factor). β actin was used as control in case of cytoplasmic fractions used. For nuclear and mitochondrial fraction equal loading of protein was checked by Ponceau staining (not shown in figure). Protein was estimated in these fractions according to Lowry's method [25]. Protein was resolved on polyacrylamide gel (acrylamide:bis-acrylamide 19:1) containing 0.1% SDS (SDS-PAGE). For cytochrome *c*, 20 μ g of protein was taken and the concentration of gel was 10%, while in case of AIF 40 μ g of protein was taken and the concentration of gel was 15%. After running through the resolving gel the proteins were transferred into nitrocellulose paper (Hybond ECL, Amersham Biosciences, UK) and blocked with blocking solution (5% BSA in 25 mM Tris, 0.15 M NaCl, TBS) for 2 hours at room temperature in an orbital shaker with constant shaking. TBS with 0.05% tween-20 (TBS-T) was taken for dilution of anti-cytochrome *c* (1:2000 dilution), anti- β actin (1:2500 dilution) and anti-AIF (1:1000 dilution) and was reacted with the membrane containing proteins overnight at 4°C. Next day the blots were washed with 5.0 ml TBS-T four times, followed by horseradish peroxidase-conjugated anti-mouse immunoglobulin G (1:2000 dilutions in TBS-T) for cytochrome *c* and β actin and with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (1:2000 dilution in TBS-T) for AIF, treatment for 2½ hours at room temperature. Again the membrane was washed with TBS-T four times and treated with ECL western blotting detection reagents (Amersham Biosciences) for visualization of the protein bands. The blots were scanned and intensities were recorded using Scion image software. For each assay the experiments were repeated thrice.

3. Results

3.1. Apoptotic cell morphology using Hoechst staining

The number of apoptotic cells produced by γ irradiation was visualized under fluorescent microscope in both V79 and V79^C cells. The morphology of the nuclei was examined after staining with the DNA binding dye Hoechst 33258. Typical snapshots of such cells are shown in fig. 1(a-d). Both cell types did not show the typical morphology of nuclear fragmentation in the untreated condition. Irradiation with γ resulted in significant apoptotic induction in both cell types ($p < 0.01$) although a difference in the extent of this induction was observed. V79 cells showed 38.80 ± 0.28 % of cells to be apoptotic, at the same dose of 6 Gy, V79^C cells had only 20.260 ± 0.037 % apoptotic cells that was significantly lower. This is shown in the bar diagram 1e. The experiment was repeated thrice.

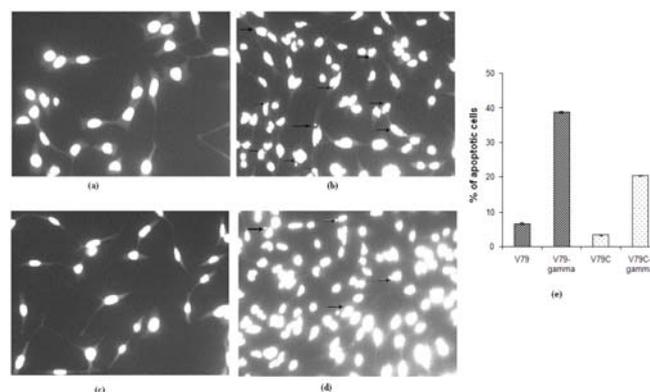


Figure 1: Formation of apoptotic bodies in cells irradiated with γ -rays (6 Gy), observed after staining with Hoechst 33258 under a fluorescent microscope in (a) V79 control cells, (b) V79^C control cells, (c) γ irradiated V79 cells, (d) γ irradiated V79^C cells and (e) bar diagram showing percentage of apoptotic cells; 1- V79 control, 2- γ irradiated V79 cells, 3- V79^C cells, 4- γ irradiated V79^C cells. At least 200 cells each were scored.

3.2. Nucleosomal ladder formation upon γ irradiation in V79 and V79^C cells

Fig. 2 shows the results of a typical experiment showing the formation of nucleosomal ladder in V79 and V79^C cells respectively after 6 Gy of γ ray exposure. It is evident from the figure that apoptosis induced in the conditioned cells after γ irradiation was significantly lower than that in V79 cells.

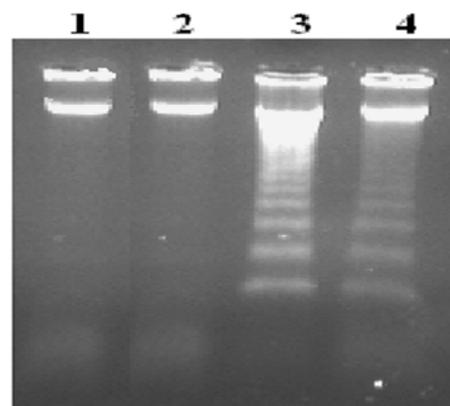


Figure 2: Formation of a typical DNA ladder in agarose gel upon γ irradiation of cells; lane 1- V79 control cells; lane 2- V79^C control cells; lane 3- γ irradiated DNA of V79 cells; lane 4- γ irradiated DNA of V79^C cells.

3.3. Cytochrome c protein expression in γ irradiated V79 and V79^C cells

Fig. 3a shows a typical Western blot of the cytochrome *c* released into the cytoplasm in the conditioned cells both before and after γ -irradiation (6 Gy) in comparison to its parental cells. β actin expression was used as the standard. Cytochrome *c* content in the cytoplasm of unirradiated V79 cells was arbitrarily taken as unity. Compared to this, the amount of cytochrome *c* present in the cytoplasm of V79^C cells was significantly lowered ($p < 0.01$). Upon γ irradiation there was a marked increase in the release of this protein into the cytoplasm of the parental V79 cells; but, there was no

significant increase in the release of this protein in V79^C cells. The lower cytoplasmic content of cytochrome *c* in irradiated V79^C cells accounted for lower apoptotic yield in these cells. The mean of the results from two such experiments is shown by a bar diagram in fig. 3b.

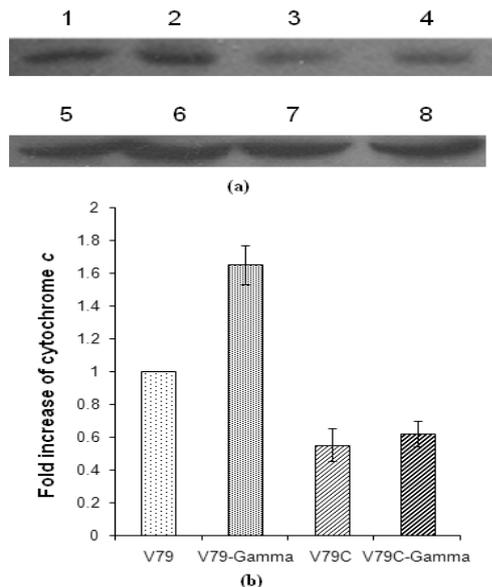


Figure 3: (a) Cytochrome *c* released in the cytoplasmic fractions of V79 and V79^C cells after 6Gy of γ -irradiation. Lanes from left to right are for unirradiated V79, irradiated V79, unirradiated V79^C and irradiated V79^C cells respectively. The β actins present in the corresponding cytoplasmic fractions are shown in the second row in the same order. (b) The result in terms of fold increase of cytoplasmic cytochrome *c* in unirradiated V79, irradiated V79, unirradiated V79^C and irradiated V79^C cells are shown in the bar diagram in this order respectively. The amount of cytochrome *c* present in the cytoplasm of unirradiated V79 cells was arbitrarily taken as unity for calculation of fold increases. [* $p < 0.01$] This is the mean + S.D. from two experiments.

3.4. Assay of caspase 9 activity in unirradiated and γ irradiated V79 and V79^C cells

Fig. 4 shows a comparison of the caspase 9 activity in V79^C cells with respect to that in V79 cells. In unirradiated controls, the conditioned cells had 0.59 fold of the activity found in parental cells ($p < 0.05$). Upon irradiation with 6 Gy of γ rays, there was a significant increase of caspase 9 activities in V79 cells ($p < 0.05$). In contrast, the change in caspase 9 activities in V79^C cells upon irradiation was not significant.

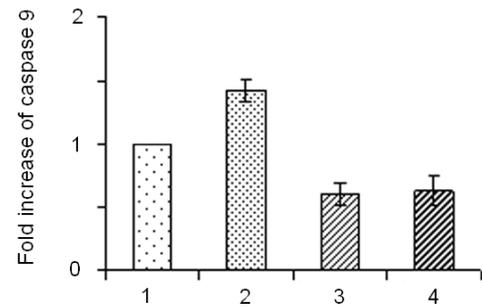


Figure 4: The fold increase in caspase 9 activities found on γ irradiation of cells. The dotted bars are for V79 cells and striped bars are for V79^C cells. 1 & 3- control; 2 & 4- γ irradiated.

3.5. Assay of caspase 3 activity in unirradiated and γ irradiated V79 and V79^C cells

Fig. 5 shows a comparison of the caspase 3 activity in V79^C cells with respect to that in V79 cells. In untreated control V79^C cells, the activity of caspase 3 was 0.60 times of that found in the V79 cells ($p < 0.05$). 6 Gy of γ irradiation resulted in a significant ($p < 0.01$) increase of caspase 3 activity in V79 cells, but there was no significant change in caspase 3 activity in V79^C cells.

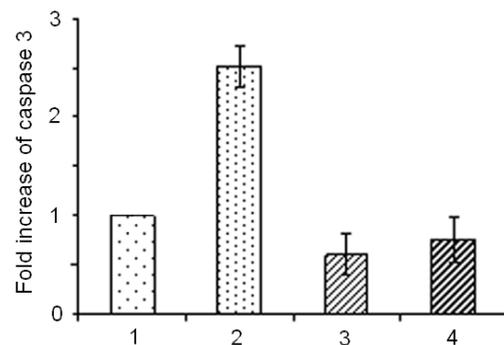


Figure 5: The fold increase in caspase 3 activities found on γ irradiation of cells. The dotted bars are for V79 cells and striped bars are for V79^C cells. 1 & 3- control; 2 & 4- γ irradiated

3.6. AIF protein expression in unirradiated and γ irradiated V79 and V79^C cells

AIF protein was detected in both the nuclear and cytoplasmic fraction. The result of a typical experiment is shown in fig. 6a. Fig. 6b show the fold increases of AIF in the mitochondrial and nuclear fraction for control and γ irradiated cells of both types. There was no significant difference in the AIF protein found in the mitochondria as well as in the nucleus of unirradiated V79 and V79^C cells. Upon γ irradiation there was a significant increase in AIF in the nuclear fractions of both cell types ($p < 0.01$). The difference in the amount of AIF detected both in the mitochondrial and the nuclear fractions of irradiated cells, in each cell types was not significant. Thus, caspase-independent apoptosis mediated through AIF is not modulated in these conditioned cells.

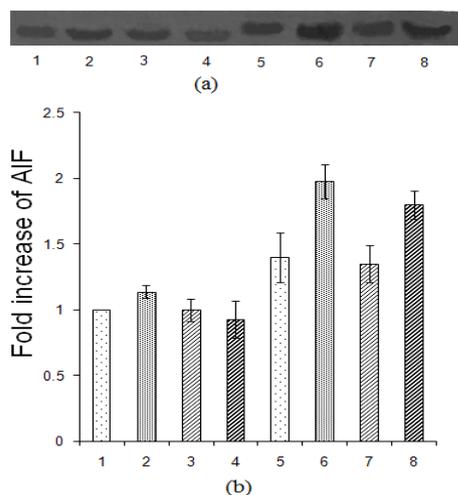


Figure 6: (a) AIF found in the mitochondrial and nuclear fraction on γ irradiation of cells. Lanes 1-4 are the mitochondrial fractions and lanes 5-8 are for the nuclear fractions respectively. Lanes 1 & 5 in V79 control; lanes 2 & 6 in γ irradiated V79; lanes 3 & 7 in V79^C control, and lanes 4 & 8 in γ irradiated V79^C cells. (b) The bar diagram depicts the fold increase/decrease in AIF. The results are mean \pm S.D from 3 experiments. The bars marked 1-8 are in the same order as the lanes 1-8 in (a).

4. Discussion

Our earlier studies had revealed that cells conditioned with low doses of H₂O₂ were cross-resistant to γ rays [7]. This resistance could be due to inhibition of apoptotic cell killing in these conditioned V79^C cells as evidenced through nucleosomal ladder formation and Hoechst staining of cell. Several investigators have shown that enhanced antioxidant defense can lead to resistance toward apoptotic induction [9]-[11]. Our present finding corroborates this, as our earlier observations had revealed that the conditioned V79^C cells intrinsically had elevated antioxidant enzyme activities that were increased even further upon exposure to these damaging agents [8].

Apoptosis plays a critical role not only in development of cancer, but also in the response of cancer to anticancer agents. Mitochondria in particular are known to play an important role in the induction of apoptosis. On the other hand, mitochondrial dysfunction is one of the commonest and consistent phenotype of cancer [26], [27]. Considering that most tumor cells are resistant to apoptosis, it is likely that resistance to anticancer agents is related to the particular properties of mitochondria in cancer cells that are distinct from mitochondria of normal cells. Over-expression of mitochondrial genes is also often observed in highly metastatic cancer cells [28], [29]. Earlier we have shown that the mitochondrial genes ND1 and ND4, subunits of NADH dehydrogenase enzyme are over-expressed in these cells [30].

A number of pro-apoptotic proteins remain secure within the inter-membrane space of the mitochondria that are released only upon appropriate signal to trigger apoptosis. Cytochrome *c* is one such protein that normally remains secured behind the outer mitochondrial membrane [14]. Catalase over-expressing cells have been found to

demonstrate delay or lack of cytochrome *c* released from mitochondria [9], [11]. Our present findings showed that the significantly low cytoplasmic cytochrome *c* level in the V79^C cells could be responsible for lower apoptotic death. We have also shown earlier that V79^C cells have considerably higher catalase activity compared to their parental cells [30].

Cytochrome *c* triggers the proteolytic maturation of caspases with the formation of apoptosome, which is an active complex that includes Apaf-1 and caspase 9 [31], [32]. Caspase 9 is an important downstream protease that plays an important role in apoptotic induction through a number of different pathways [31]-[34]. For unirradiated controls, the activity of caspase 9 was highly reduced in the conditioned cells. On irradiation, in contrast to an enhancement in its activity in V79 cells, there was no change in V79^C cells. Thus, it appears that all apoptotic pathways involving caspase 9 were inhibited in these cells.

DNA fragmentation is a key feature of apoptosis, characterized by the activation of some endogenous endonucleases, the caspase activated DNases (CAD) that cleave chromatin DNA into internucleosomal fragments [35]. Caspase 3 is a downstream caspase that activates some nucleases. These CAD molecules preexist as an inactive complex with an inhibitory subunit. The activation of CAD occurs through activation of caspase 3-mediated cleavage of the inhibitor CAD that results in the release and activation of the catalytic subunit [33], [34]. Ionizing radiation induces apoptosis through caspase3-dependent pathway [36]. The inability of the conditioned V79^C cells to activate caspase 3 indicated the suppression of caspase 3-dependent apoptotic cell death. Our results thus demonstrated that cells exposed to chronic low doses of oxidant were resistant to γ rays because of inhibition of caspase-dependent apoptotic cell killing.

Apoptosis can also be triggered through caspase independent pathway. One such factor is apoptosis inducing factor (AIF) [17], [35], [37]. It is phylogenetically a flavoprotein, which is confined into the mitochondrial intermembrane space. Upon appropriate signal it translocates via cytosol to the nucleus where it binds to DNA and provokes caspase-independent chromatin condensation. Besides the lethal function of AIF, it also contributes to cell vitality via its redox function and is central for optimal oxidative phosphorylation and for effective antioxidant defense [38]. AIF is also known to make crucial contribution to the biogenesis and/or maintenance of the NADH dehydrogenase complex of the mitochondrial electron transport chain [39]. As we have found expression of subunits of the NADH dehydrogenase complex to be altered in these cells, it was necessary to see if this could affect AIF-mediated apoptosis. The AIF content did not differ significantly in the mitochondrial and nuclear fraction in each cell types, for both irradiated and unirradiated condition. Thus caspase independent pathway of apoptosis was not affected in these cells.

5. Conclusion

In conclusion, we can say that in cells exposed to chronic doses of oxidants apoptotic death is suppressed. This inhibition of apoptosis is related to suppression of caspase-mediated cell death pathways but not that involving the AIF. These findings are important as they may aid in understanding the mechanism of resistance in tumors arising from oxidative stress. Radio-resistance in such cells may be surmounted by supplementation with suitable caspases.

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