Note

Effect of Therapeutic Doses of Ionising Radiation on the Somatomammotroph Pituitary Cell Line, GH3

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Abstract. Ionising radiation is used for the treatment of pituitary tumours as fractionated radiotherapy, where the total dose reaching the tumour area is in the range of 40–50 Gy, or during stereotactic radiosurgery, where the total dose reaching the tumour area during one session is in the range of 20–90 Gy. In this study, we investigated the effect of ionising radiation of 60Co (dose rate of 3 Gy/min, similar to that used during gamma knife procedure) on the mode of cell death induced by irradiation of this GH3 cell line by γ-rays was programmed cell death – apoptosis. Doses of 20–50 Gy were shown to inhibit proliferation in these cells. 24 hours after irradiation with a dose of 20 and 50 Gy, cells were shown to accumulate in the G2/M phase of cell cycle. This cell cycle arrest lasted for at least ten days. Apoptosis was detected 72 hours towards until the end of the study (10 days). However, a significant number of cells were still alive ten days following irradiation. We conclude that ionising radiation doses of 20 and 50 Gy induce pituitary GH3 cell apoptosis following cell cycle arrest in the G2/M phase.

Key words: Apoptosis, Ionising radiation, Pituitary cells GH3


Acromegaly is a debilitating disorder that usually develops over many years and is caused by long-term hypersecretion of growth hormone (GH), in the vast majority of cases, from a benign pituitary adenoma [1]. Surgery remains the treatment of choice for most patients [2], however only 70–80% of patients with microadenomas and 50–60% of patients with macroadenomas can sufficient control of disease activity be achieved [2–5]. Somatostatin analogues are used for acromegaly treatment both following surgery [6, 7] and as a primary therapy [8], however life-long therapy is both inconvenient and very expensive. For this reason, radiotherapy has been widely used as adjuvant treatment for residual adenomas [2]. In the case of conventional and heavy particle (proton beam) irradiation, the total dose reaching the tumour area is in the range of 40–50 Gy. It may take up to 20 years of therapy for 90% of patients to obtain GH levels less than 5 μg/l [2]. Gamma knife radiosurgery delivers up to 70 Gy to the center of the adenoma. Normalisation of hormone secretion following this procedure is reached in 1–2 years [11, 12].

Exposure of cells to ionising radiation leads to cellular damage primarily through a spectrum of lesions in the DNA, of which double-strand breaks represent the most lethal form of damage. These lesions lead to the expression of a number of proteins, including DNA dependent protein kinases, especially ataxia-telangiectasia-mutated (ATM) kinase. Activ-
Induction of apoptosis by ionising radiation has been studied in many different cell types and it has been found that both the mechanism of the effect and its time dependence are significantly different in particular cell types [17]. In cell lines, where p53 is mutated or missing, apoptosis is induced after cell cycle arrest in the G2 phase. In our previous study [18], we demonstrated that irradiation of cells of human promyelocytic leukaemia HL-60 (p53 is absent) induces two types of cell death in dose-dependent manners. In interphase death or premitotic apoptosis (after doses of 20–100 Gy), cells die by apoptosis at all phases of the cell cycle, and in reproductive or mitotic cell death (mitosis related apoptosis) following lower doses, cells die by apoptosis after the G2/M arrest or mitosis attempt.

In our previous studies of human promyelocytic leukemia cells HL-60, three different methods were used for the detection of apoptosis [19]: 1) morphological methods of evaluation of Diff-Quick stained cytospin preparations; 2) flow cytometric detection of subG1 DNA content; and 3) mitochondrial membrane protein specific monoclonal antibody, APO2.7 with and without permeabilization with digitonin. Six hours after irradiation with a dose of 10 Gy, we observed only a minimal percentage of apoptotic cells by using all above mentioned methods (morphological method – 11.1%, subG1 DNA – 11.7% and digitonin-processed stained cells with APO2.7-PE – 6.1%). Significant apoptosis occurred six hours after irradiation with a dose of 20 Gy (morphological method – 49%, subG1 DNA – 32.4% and digitonin-processed stained cells with APO2.7-PE – 32.7%). Our conclusions are in a good agreement with the data of Hopcia et al. [20] and Dynlacht et al. [21]. Six hours post-irradiation with a dose of 20 Gy cleavage of lamin B – 70 kDa to 46 kDa fragment was detected in both the cytosol and nucleus using Western blot analysis. After a dose of 8 Gy, the 46 kDa fragment was detected only 24 hours after irradiation, also in the cytosol and nucleus. Results of this method are in good correlation with the results of morphological methods and flow-cytometric subG1 peak detection; in which lamin B cleavage was found to precede the subG1 peak [22].

Cells of human T-lymphocytic leukemia MOLT-4 exhibit a wide dispersity in the timing of induction and execution of radiation-induced cell death that includes rapid-interphase apoptosis, delayed apoptosis and postmitotic apoptosis. Following irradiation with a dose of 7.5, Gy lamin B cleavage was detected six hours after irradiation and in this time, apoptosis was also detected using subG1 peak increase and detection of APOP2.7 after digitonin permeabilization. MOLT-4 cells are highly radiosensitive (D0 = 0.9 Gy) and possess p53 wild/mutant genes. MOLT-4 cells undergo apoptosis following irradiation via the p53-dependent pathway [23]. In contrast, HL-60 cells are p53 negative, and after low doses of up to 8 Gy exhibit strong cell cycle arrest in the G2/M phase, where they repair damage caused by irradiation. In the case of ineffective repair apoptosis is induced to a maximum within 48–72 hours following irradiation. HL-60 cells are more radioresistant then MOLT-4 cells, D0 = 2.2 Gy. Radford [24] presumes that the central role in ionizing radiation-induced apoptosis has been the protein triad p53, PARP-1 and topoisomerase I and that the radiosensitivity is closely related with p53 status.

The somatotroph cell line, GH3 is derived from a rat pituitary adenoma that produces growth hormone and prolactin. Yin et al. [25] described the increase of wild type p53 accompanying bromocryptine induced apoptosis in GH3 cells. In our study, we investigated the relationship between apoptosis induction and cell cycle arrest in the G1 or G2 phase of GH3 cells irradiated by 20 and 50 Gy of ionising gamma radiation.
Materials and methods

Cell culture

The somatomammotroph cell line, GH3 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). GH3 cells were cultured in F10-Ham medium (Sigma-Aldrich) supplemented with 15% horse serum (Sigma-Aldrich), 2% foetal bovine serum (Sigma-Aldrich) and 2 nM glutamine (Sevac, Prague, Czech Rep.) in a humidified 37°C incubator with a controlled 5% CO₂ atmosphere. Cultures were divided after forming a monolayer by harvesting and diluting to a concentration of 5 × 10⁵ cells/ml. Cell counts were performed with a hemocytometer, cell membrane integrity was determined using the Trypan blue exclusion technique. GH3 cells up to ten passages were used for this study.

Gamma irradiation

Exponentially-growing GH3 cells were suspended at a concentration of 5 × 10⁵ cells/ml in complete medium. 10 ml aliquots were plated into 25 cm² flasks (Nunc) and irradiated at room temperature using a ⁶⁰Co γ-ray source at a dose rate of 3 Gy/min. After irradiation, flasks were placed in a 37°C incubator and removed at various times following irradiation for analysis. The cells were counted and cell viability was determined by a Trypan blue exclusion assay.

Cell cycle analysis

Following incubation, cells were washed with cold PBS and fixed with 70% ethanol. For fixation of low molecular-weight fragments of DNA the cells were incubated for 5 min at room temperature in buffer (192 ml 0.2 M Na₂HPO₄ + 8 ml 0.1 M citric acid, pH7.8) and then stained with propidium iodide (PI) in Vindelov’s solution for 30 minutes at 37°C. Fluorescence (DNA content) was measured with a Coulter Electronic (Hialeah, FL, USA) flow cytometric apparatus. A minimum of 1 × 10⁴ cells analysed in each sample served to determine the percentage of cells in each phase of the cell cycle, using a Multi-cycle AV software. A minimum of three independent experiments were performed.

Cell morphology

To calculate the percentage of cells showing an apoptotic morphology cell aliquots were removed from control and irradiated cell cultures at various times of incubation and usually 400 cells were counted on Diff-Quik-stained cytospin preparations (Dade Behring, Switzerland). Apoptotic cells were identified by the condensed and fragmented state of their nuclei and focal protrusions on the cell surface.

Colony assay

GH3 cells were grown in 25 cm² cultivation flasks in complete medium. All cultures were performed in duplicate. Colonies of more than 40 cells were counted after 21 days of incubation in 5% CO₂ at 37°C. Dose response curves of GH3 cells irradiated in vitro by increasing doses of 0.5–10 Gy were used for D₀ value calculations.

Statistical analysis

The results were statistically evaluated with a Student’s t-test. The values represent mean ± SD (standard deviation of the mean). Statistical significance of the difference of means in comparable sets is indicated.

Results

Cell growth

Fig. 1 shows the effects of irradiation by doses of 20 and 50 Gy on the proliferative rate of the GH3 cell line. At the beginning of the experiment, cells were diluted to a concentration of 5 × 10⁵/ml. At that stage, exponential growth of control cells was apparent during the observation interval (Fig. 1.). Both doses of irradiation induced significant inhibition of proliferation (p<0.01 by Student’s t-test) in comparison to the control, nonirradiated group. However, even after ten days following irradiation, a significant number of cells were found to be alive as determined by a Trypan blue exclusion assay (20 Gy: 43.7 ± 4.0 × 10⁴ cell/ml; 50 Gy: 44 ± 2.8 × 10⁴ cell/ml).
Cell cycle

From Fig. 2 it is apparent that following irradiation of GH3 cells at both doses, 20 and 50 Gy, the cells were shown to undergo cell cycle arrest in the G<sub>2</sub>/M phase, which was observed 24 hours after irradiation and lasted during the whole experiment (ten days following irradiation). The intensity of the cell cycle arrest in the G<sub>2</sub>/M phase was shown to be dose- and time-dependent (Figs. 2 and 3).

Apoptosis

Apoptosis was detected using two different methods — DNA fragmentation detected by flow cytometric DNA analysis and morphological changes of cells detected on Diff-Quik-stained cytopsin preparations.

A significant amount of apoptotic cells with sub-diploid DNA (i.e. subG<sub>1</sub> peak) was detected 72 hours after irradiation and increased throughout the experiment (ten days following irradiation). The correlation between the G<sub>2</sub> phase arrest and apoptosis after 50 Gy irradiation of GH3 cells is shown in Fig. 4.

Evaluation of morphologic changes of the irradiated cells also confirmed induction of apoptosis-apoptotic cells with condensed and fragmented nuclei were detected.

Radiosensitivity

Fig. 5 shows the dose response curve for GH3 cells. It has been found that despite a long G<sub>2</sub> phase arrest and delayed apoptosis after high doses (20 and 50 Gy) the D<sub>0</sub> value for GH3 cells was 2.5 Gy.

Discussion

The induction of apoptosis studied in pituitary cells GH3 (rat somatomamotroph) and AtT-20 following exposure to bromocryptin (mouse corticotroph cell line) has been previously reported [25]. In another report, p38 MAP kinase is activated in GH3 cells during bromocryptine-induced apoptosis [26]. The apoptosis of GH3 cells was also observed following treatment with a dopaminergic neurotoxin, 1-methyl-4-phenylpyridinium ion [27], and with inhibitors of serine/threonine phosphatases 1 and 2A [28, 29].

In most cases of acromegaly, initial therapy should be surgical. However, good control of disease activity (GH<2.5 μg/l) can be achieved by surgery alone in only approximately 60% of acromegalic patients [3], and additional treatment is often necessary. Ionising radiation has been used to treat acromegaly for many years. It is generally accepted that pituitary tumours respond to ionising radiation therapy and such therapy can lead to disease-free remission. Following radiotherapy in acromegalic patients IGF-1 and growth
hormone concentrations decrease to normal levels in 70–90% of patients with a decrease rate of 10–30% per year [9]. With the use of a gamma knife, the median latency is shorter: 12–15 months [11, 12]. The cause for this difference between classic and focal irradiation is not known. While the cumulative doses are comparable, the gamma knife dose rates are incomparably higher. However, little is known about the mechanism of effect of ionising \( \gamma \)-radiation on endocrine pituitary cells at the cellular level.

Ionising radiation is a well-known DNA damaging agent. These radiation-induced lesions lead to the activation of an apoptotic cascade and affect cell cycle checkpoint mechanisms, which have been studied in many different cell lines. Accumulating data suggest that ATM kinase is a proximal component of the DNA damage-induced cell cycle checkpoint pathway [30], as well as apoptosis induction.

Leukemic cell lines are usually very sensitive to induction of apoptosis by ionising radiation. Postmitotic apoptosis in the HL-60 cell line (TP53 negative) was observed after the G2 phase arrest of the cell cycle 72 hours (maximum) following irradiation by doses of 2–10 Gy. Cells of human T-lymphocytic leukemia MOLT-4 (TP53 wild/mutant) undergo apoptosis much more quickly. The number of cells in the G2 phase increased moderately and 80% of cells were apoptotic 24 hours after irradiation with a dose of 5 Gy. Cells of pituitary origin are less sensitive to ionising radiation (GH3 cell line \( D_0 = 2.5 \) Gy) then cells of leukemic lines. The effect of \( \gamma \)-radiation (25 Gy) on the pituitary corticotroph cell line, AtT-20 (TP53 wild type) was studied by Woloschak et al. [31]. They demonstrated that a significant proportion of these cells underwent apoptosis within 48 hours of the initial irradiation. Woloschak et al. also found that there were increased proportions of cells in both the

![Fig. 2. Dynamic changes of cell-cycle and apoptosis induction after irradiation of GH3 cells by 20 and 50 Gy of ionising radiation. Flow-cytometric analysis of DNA content and cell cycle after irradiation of GH3 cells by increasing doses of ionising radiation. Cell cycle analysis was performed using propidium iodide staining of DNA. Apoptotic cells were identified as cells with subdiploid DNA content i.e. subG1 peak. Representative results for a single experiment are shown.](#)

![Fig. 3. Flow-cytometric analysis of DNA content and cell cycle 48 hours after irradiation of GH3 cells by increasing doses of ionizing radiation. Cell cycle analysis was performed using propidium iodide staining of DNA. Apoptotic cells were identified as cells with subdiploid DNA content i.e. subG1 peak. Representative results for a single experiment are shown. Premitotic apoptosis could be seen only after an extremely high dose (200 Gy).](#)
G₁ and G₂ phase of the cell cycle. The cells were all dead within 4–5 days. In a previous study [32], using AtT-20/D16v-F2 (adherent subclone of AtT-20 cells, TP53 wild type), we observed complete inhibition of cell proliferation after irradiation with doses of 10–100 Gy. A dose of 5 Gy induced a decrease in the proliferation rate within 168 hours after irradiation. After irradiation with a dose of 10 Gy a decrease in the number of cells in the S phase of the cell cycle was apparent, which correlates with growth inhibition. Apoptosis was apparent 24–48 hours after irradiation with a dose of 10 Gy, and at 96 hours the cells were
In this study, we examined the effect of usual therapeutic doses of ionising γ-radiation of 20 and 50 Gy, administered at a dose rate similar to the gamma knife procedure to somatomammotroph rat cells GH3. Strong cell cycle arrest in the G2/M phase starting 24 hours after irradiation and lasting for the entirety of the experiment (10 days) was observed. During apoptosis, chromosomal DNA is cleaved by endonucleases into nucleosome-sized pieces of about 180 base pairs. These fragments leak out of the cell and the DNA content of apoptotic cells is therefore lower than the DNA content of cells in the G1 phase of the cell cycle. 48 hours after irradiation, the population of GH3 cells with a DNA amount lower then those in the G1 phase was observed (subG1 peak). The number of apoptotic cells increased until ten days after irradiation (the end of the experiment). Morphological changes of irradiated GH3 cells confirmed the occurrence of apoptosis. However, despite D0 value 2.5 Gy, even ten days after irradiation with doses of 20 and 50 Gy, a significant number of GH3 cells were found to be still alive. Despite lower proliferative activity of GH3 cells in comparison to leukemic cell lines, GH3 cells are much more proliferative than human somatotroph adenoma cells. It is generally accepted that cells with a high proliferative activity are more radiosensitive; therefore radiosensitivity of human somatotroph adenoma cells could be even lower.

It could be concluded, that apoptosis is induced by therapeutic doses of ionising radiation in GH3 cells administered in a dose-rate similar to that used during gamma knife radiosurgery. In contrast to the human promyelocytic leukemia cell line HL-60, where after irradiation with doses of 20 and 50 Gy quick (6 hours after irradiation) premitotic apoptosis was induced from all phases of the cell cycle, GH3 cells enter into a long G2 phase cell cycle arrest and go into postmitotic apoptosis only from this phase of the cell cycle. Long G2 phase cell cycle arrest, during which the cells attempt to repair DNA damage is usually observed in cells without a functional p53 (TP53 missing or mutated). Further studies of p53 status and its phosphorylation in GH3 cells are necessary. Further studies are required to elucidate the possible involvement of long G2 phase cell cycle arrest and postmitotic apoptosis in the slow decrease of secretory activity of acromegalic adenomas after radiotherapy and to evaluate the effect of substances preventing G2 phase entry after irradiation (such as caffeine), which should prevent damage reparation and enhance apoptosis.

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References


