

Effects of Low-Dose Ionising Radiation on Pituitary Adenoma: is there a Role for L-type Calcium Channel?

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ABSTRACT

Pituitary adenomas constitute about 6–18% of brain tumours in adults. Activation of voltage gated calcium currents can account for growth hormone oversecretion in some GH-secreting pituitary adenomas that produce an acromegaly appearance and increase mortality. Ca^{2+} ions, as mediators of intracellular signalling, are crucial for the development of apoptosis. However, the role of $[Ca^{2+}]$ in the development of apoptosis is ambiguous. In this study, the effects of low-dose ionising gamma radiation (^{60}Co) on rat pituitary adenoma cells survival and proliferation and the role of calcium channels on the apoptosis radio-induced were evaluated. Doses as low as 3 Gy were found to inhibit GH3 cell proliferation. Even though there was a significant number of live cells, 168 hours following irradiation, they were not able to proliferate. The results indicate that the blockade of extracellular calcium influx through these channels does not interfere in the radiation-induced apoptosis in GH3 cells.

Key words: Calcium channels; ionising radiation; pituitary adenoma cells

INTRODUCTION

Pituitary adenomas constitute about 6–18% of brain tumours in adults (Frohman, 1995). Despite the fact that pituitary adenoma is a benign tumour, patients can develop acromegaly due to hypersecretion of growth hormone that, not only alters appearance of these patients (acromegalic appearance), but also increases mortality (Klibanski et al, 1995). Currently, only 20-50% of patients can achieve the control of hormone activity after surgery. Somatostatin analogues are used for acromegaly treatment; however, life-long therapy is both inconvenient and very expensive. For this reason, radiotherapy has been widely used as adjuvant treatment for residual adenome (Melmed, 1990).

The major risk from gamma knife radiosurgery is radiation damage to the optic chiasm. To reduce this unwanted radiation effect, searches for radioprotective agents and alternative therapies have been performed. In a search for radioprotective agents, diltiazem, a calcium antagonist with a benzothiazepine structure, was found to protect mice against a lethal gamma radiation dose (Floersheim, 1992).

The role of $[Ca^{2+}]_i$ in apoptosis induced by agents that do not immediately increase $[Ca^{2+}]_i$, such as 5-FdU, TGF beta-1, doxorubicin, or radiation, is far more controversial (Tombal et al, 2002). $[Ca^{2+}]_i$ homeostasis is achieved by intracytoplasmic Ca^{2+} stores (Endoplasmic Reticulum and mitochondria) and Ca^{2+} influx through Ca^{2+} channels. In pituitary adenoma cells, voltage-dependent Ca^{2+} channels (Ca_v) regulate the

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Ca²⁺ influx responsible for hormone secretion. In a previous report (Lomeo et al, 2004), the presence of Ca_v1 (L-type Ca_v) in GH3 cells, an immortalized cell line derived from a rat pituitary adenoma, was shown. Calcium is required as a cofactor by primer recognition proteins involved in DNA synthesis and by protein kinase C (PKC), which is activated by ionising radiation (Hallahan et al, 1994). However, the role of Ca²⁺ influx through Cav1 on the radiation effects has not been evaluated. Because these processes may be involved in radiation-mediated regulation of the progression of cells through the phases of the cell cycle, the effects of the Ca_v1 antagonists on the pituitary adenoma radio-induced cell death were studied.

MATERIALS AND METHODS

Cell Culture

GH3 cells from rat pituitary adenoma (ATCC, Rockville, MD, USA) were grown in a humidified atmosphere of 5% CO₂/95% air, at 37 °C. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 0.1 % antibiotics.

Gamma Irradiation

GH3 cells were irradiated at a rate of 0.3 Gy/minute in a GammaCell irradiator (⁶⁰Co; LIG, CDTN/CNEN, Brazil) at room temperature in the presence of molecular oxygen. Following irradiation, cells were incubated at 37 °C.

Calcium channel antagonist

Nifedipine, a dihydropyridine calcium antagonist, was used as calcium channel blocker (5µM). The cells were incubated with Nifedipine one hour before irradiation (3Gy). Nifedipine was replaced every 24 hours.

Growth-inhibition Assay

Exponentially growing GH3 cells were plated in 40 mm Petri dishes at 10⁵ cells/dish. After attachment, cells were gamma irradiated (IR, 3-12 Gy) in the presence or absence of Nifedipine (5µM). After exposure to IR, the cells were returned to the CO₂ incubator. Cell cytotoxicity was determined by evaluation of the membrane integrity after different times using Trypan blue exclusion.

Metabolic viability assay

GH3 cells (0.5x10⁴ cells/well) were plated in 24 well dishes and treated with gamma radiation at 3-12 Gy. Cellular metabolic viability was measured photometrically after 24, 48, 144 and 216 hours by MTT assay.

Clonogenic assay

GH3 cells were initially plated in 60 mm-Petri dishes at a density of 10⁴ cells per dish and allowed to attach overnight. Cells were gamma irradiated at 3-12 Gy and incubated for colony formation. After formation of colonies, they were stained with Giemsa. Colonies with more than 50 cells were counted. The loss of clonogenic survival was determined from the ratio of colonies in the treated cell group/colonies in the control group. All of the experiments were done in triplicate and performed at least three times.

Statistical analysis

Data were expressed as means ± S.D. Statistical significance of differences between means was determined by Student's t test. Differences were considered significant at the level $p < 0.05$.

RESULTS AND DISCUSSION

Ionising radiation has been used to treat acromegaly for many years. In general, pituitary tumours respond to ionising radiation therapy and such therapy can lead to disease-free remission.

Following radiotherapy in acromegalic patients, hormone concentrations decrease to normal levels in ~80% of the patients, with a decrease rate of ~20% per year (Becker et al, 2002). With the use of the gamma knife, the median latency is 12-15 months shorter (Vladyka et al, 2000). The mechanisms involved in this difference between classic and focal irradiation are not known. In addition, the knowledge about the mechanism of the effect of ionising gamma radiation on endocrine pituitary cells is not completely established. In this study the effect of low dose ionising radiation on GH3 cells and the involvement of Ca_v1 was examined.

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.

The effect of gamma irradiation by doses of 3 to 12 Gy on the cellular viability of GH3 cells is shown in Fig.1. After 24 hours, plates exposed to three Gy showed only 52.84% of viable cells, which were not altered significantly after this time. At six Gy, only 27.97% of viable cells were found

after 24 hours. This effect progressed to 11.31% after 168 hours. Those cells exposed to twelve Gy showed 34.19% of viability after 24 hours, and progressed quickly to 2.28% after 168 hours. The treatment with gamma radiation also altered significantly the metabolic viability of GH3 cells. This effect was time- and dose-dependent.

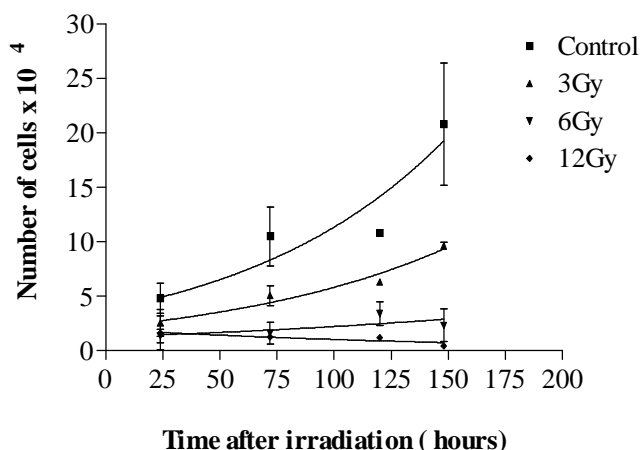


Figure 1 - Kinetics of the cytotoxic effect of gamma radiation on GH3 cell line. GH3 cells were exposed to 3-12 Gy of ionising gamma radiation. Numbers of viable cells were determined by trypan blue staining. Cytotoxic effect was dose-dependent. Only a few cells remained alive after 12 Gy. (n=3)

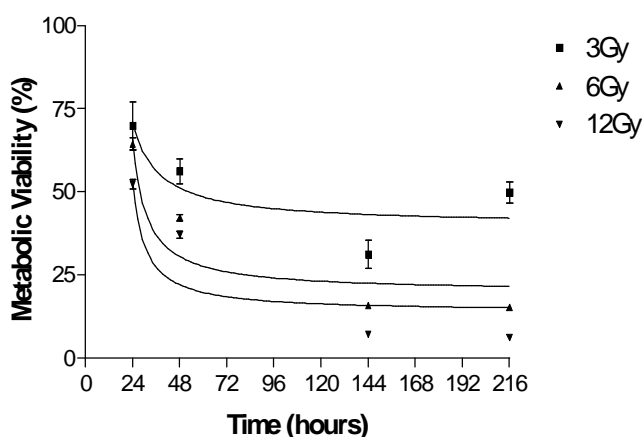


Figure 2 - Kinetics of the effect of gamma radiation on the metabolic viability of GH3 cell line. GH3 cells were exposed to 3-12Gy of ionising gamma radiation. The metabolic viability was measured by MTT assay. The effect was time- and dose-dependent (n=3), $p < 0.01$.

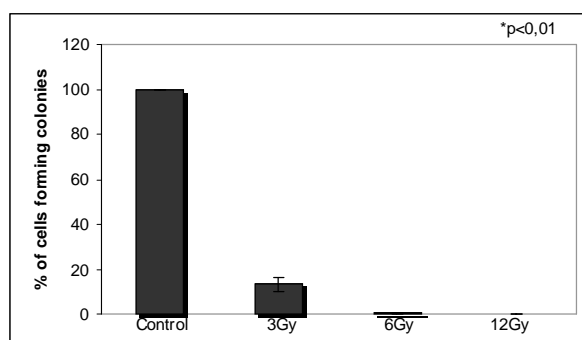


Figure 3 - Proliferation of GH3 cells exposed to gamma rays. GH3 cells were irradiated and the number of colonies was counted after 10 days of incubation. Gamma rays reduced proliferation in a dose-dependent way ($n=3$), $p<0.01$.

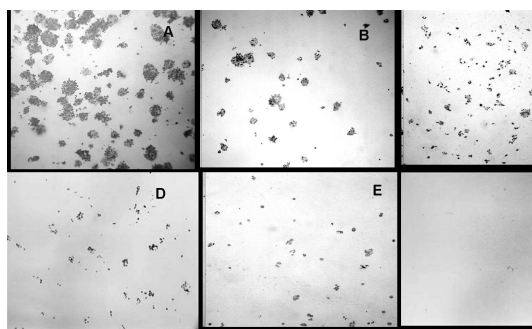


Figure 4 - Clonogenic assay. (A) control, (B) cells exposed to 3 Gy (C), cells exposed to 6 Gy, (D) cells exposed to 12 Gy, (E) cells exposed to 10^3 ng/mL of *Crotalus* venom and (F) cells exposed to 10^4 ng/mL. Cells exposed to 6 and 12 Gy of gamma radiation presented an increased cytoplasmic volume (Optic microscope: 40x).

As can be seen in Figs. 3 and 4, gamma radiation inhibited proliferation rate of GH3 cells. The cells exposed to 12 Gy did not form colonies and those ones exposed to 6 Gy showed only 0.46% of survival rate. Furthermore, the cells exposed to both doses presented an increased cytoplasmic body (Fig.4). The cells exposed to 3 Gy showed 13.22% of survival rate. It has been proposed that calcium activates endonucleases that cause DNA fragmentation and death in apoptotic cells. γ irradiation is an inducer of apoptosis that leads to increases in $[Ca^{2+}]_i$ (Story et al, 1992). Ca^{2+} ions as mediators of intracellular signaling are crucial for the development of apoptosis. An

increase in $[Ca^{2+}]_i$ of apoptosing cells (McConkey et al, 1996) due to emptying of Ca^{2+} stores and to Ca^{2+} influx from extracellular medium (Bian et al, 1997) is a general phenomenon, independent of the apoptotic stimulus. However, the role of Ca^{2+} in the development of apoptosis is ambiguous. Cell death is induced by low doses of ionising radiation in GH3 cells administered in a dose-rate similar to that used during gamma knife radiosurgery. Doses as low as 3 Gy were able to inhibit GH3 cell proliferation. Even though there was a significant number of viable cells 144 hours following irradiation, they were not able

to proliferate. The metabolic activity was also significantly decreased by gamma radiation. Nifedipine (calcium channel blocker) did not interfere significantly with the proliferative rate or with the radiation-induced cell death of GH3 cells (Fig. 5). Thus, calcium influx through Ca_v1 does not appear to be involved in this effect. These results are in agreement with those of

Marekova et al. (2003) showing that GH3 cells are radiosensitive. On the other hand, these findings differ from those of Floersheim (1992) in terms of the fundamental observation that the calcium channel antagonist does not protect γ irradiation-triggered cell death.

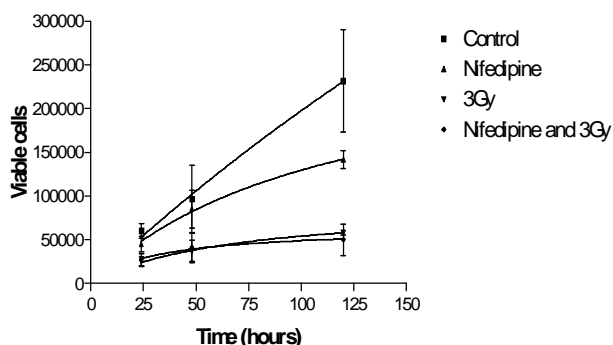


Figure 5 - Interference of Nifedipine on the radiation induced apoptosis of GH3 cells.

GH3 cells were exposed one hour to Nifedipine ($5\mu\text{M}$) and then exposed to 3 Gy of ionising gamma radiation. Numbers of viable cells were determined by trypan blue staining. Calcium channel blocker did not interfere in the proliferative rate or in the radiation induced apoptosis of GH3 cells ($n=3$).

Apoptosis of cancer cells caused by irradiation requires calcium influx by an unknown pathway (Prevarskaya et al., 2004). It has been suggested that some threshold level of cellular calcium must be reached to induce cell death (He et al., 1997). One of the numerous calcium-involving processes in mammalian cells is store operated calcium entry (SOCE) — the process in which depletion of calcium stores in the endoplasmic reticulum (ER) induces calcium influx from the extracellular space.

SOCE is part of the calcium influx stimulated by thyrotropin-releasing hormone (TRH) in GH3 pituitary cells and regulates hypophyseal secretion of prolactin (Villalobos & Garcia-Sancho, 1995). GH3 presents other calcium channels than Ca_v1 (Lomeo et al, 2004), such as Ca_v2 (Safa et al, 2001) and store-operated Ca^{2+} channels (Villalobos & Garcia-Sancho, 1995). The results obtained in this study argue against the involvement of Ca_v1 in the low dose ionising radiation effect on GH3 cells. If calcium influx from the extracellular space has a role in this effect, SOC channels can not be ruled out.

RESUMO

Adenomas de pituitária constituem cerca de 6-18% dos tumores cerebrais em adultos. A ativação de correntes de cálcio dependentes de voltagem podem levar à super-excreção de hormônio do crescimento produzindo acromegalia e aumentando a mortalidade. Íons Ca^{2+} como mediadores de sinalização intracelular são cruciais no desenvolvimento da apoptose. No entanto, o papel da $[Ca^{2+}]$ no desenvolvimento da apoptose é ambíguo. Neste estudo nós avaliamos os efeitos de baixas doses de radiação gama (^{60}Co) na sobrevivência e proliferação de células de adenoma de pituitária de rato e o papel do cálcio na apoptose radio-induzida. Nossos resultados mostraram que a dose de 3Gy foi suficiente para inibir a proliferação das células GH3. Apesar de existir um número significativo de células vivas após 168 horas do tratamento com radiação, elas não estavam aptas a proliferar. Nossos resultados também indicaram que bloqueio do influxo de

cálcio extracelular não interfere na apoptose induzida pela radiação.

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